

ROTAVIRUS INFECTIONS – DIAGNOSTIC, EPIDEMIOLOGICAL AND CLINICAL ASPECTS

By
BERTIL TUFVESSON



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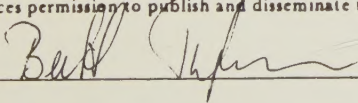


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Title and subtitle Rotavirus infections - Diagnostic, epidemiological and clinical aspects		
Abstract Occurrence of rotavirus was studied in gastroenteritis cases among infants from Malmö and Addis Ababa. Electron microscopy and immunoelectrophoresis (IEOP) were of equal sensitivity for virus detection (10 particles/ml), while enzyme-linked immunosorbent assay (ELISA) was approx. 100x more sensitive. Complement fixation and IEOP were equally sensitive for serological diagnosis and detected the infection in 96% of virus excreting infants. Serology was important to establish a diagnosis among infected adults. Secondary cases of gastroenteritis were seen in 61% of families studied, and a serological diagnosis was achieved in 52% of the adults tested. Rotavirus caused 37% of the gastroenteritis cases among children in Malmö and 28% in Addis Ababa, and was thus the most frequently isolated agent in both areas. A seasonal distribution with a peak during the coldest months every year, where rotavirus caused 62% of the gastroenteritis cases, was seen in Malmö, while a definite correlation to climate factors in Addis Ababa was not detected. The infection was most common in the age group 7-24 months in both areas. Subgroup epidemiology was studied by ELISA. In Malmö 27% were of subgroup 1, 72% of subgroup 2 and 1% of a tentative new subgroup, detected by crossed immunoelectrophoresis. The yearly distribution was stable. In Addis Ababa 33% were of subgroup 1 and 67% of subgroup 2. Subgroup 1 was more common among the less ill outpatients of ages >6 months, as compared to hospitalized patients of the same age group. Diarrhea, vomiting, fever, often preceded by respiratory symptoms, last for a week. Mild dehydration is common (45-71%). Severe dehydration occurred in 7-17%. Fatal cases due to dehydration were seen in the Ethiopian material (3%). Virus excretion is correlated to the duration of diarrhea, with a maximum on day 4-4 after onset. Rotavirus excretion was detected among infants at the maternity (13%) and neonatal unit (37%). Only 10% of the infections were symptomatic. The infections were considered endemic, with subgroup 1 occurring more frequent than among older children.		
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ROTAVIRUS INFECTIONS - DIAGNOSTIC, EPIDEMIOLOGICAL AND CLINICAL ASPECTS

AKADEMISK AVHANDLING

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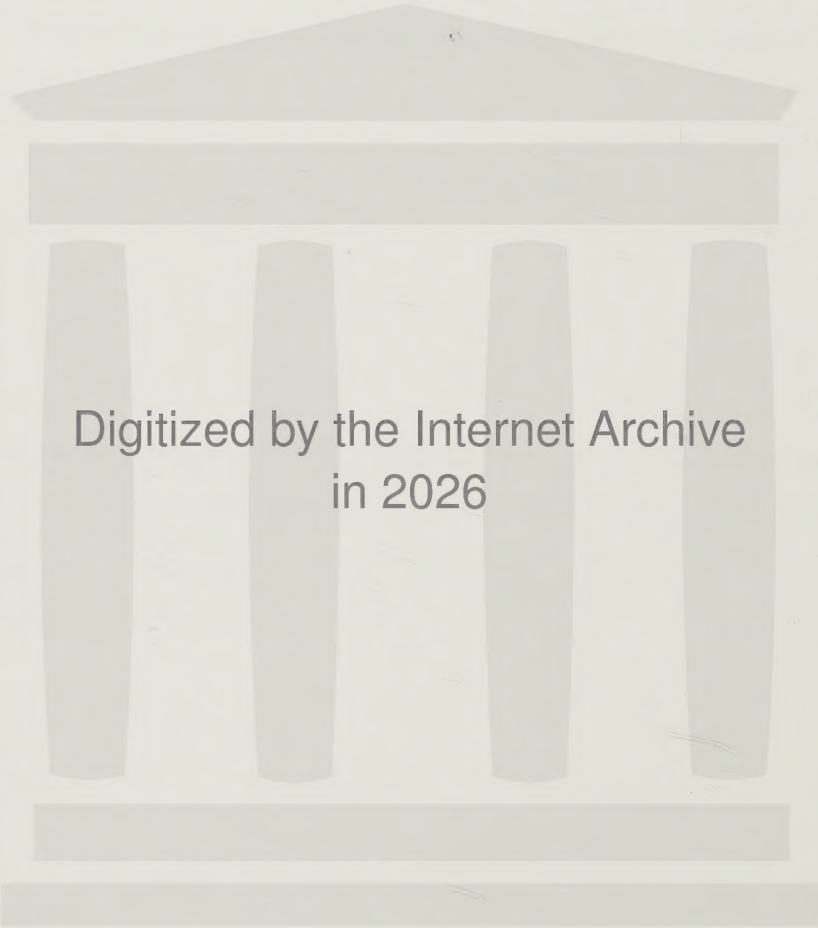
ROTAVIRUS INFECTIONS - DIAGNOSTIC, EPIDEMIOLOGICAL AND CLINICAL ASPECTS

By

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Malmö 1985



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"... men visan handlar om okända djur.

Många är långa och svåra att fånga,
många syns inte, men finns ändå.

Och de flesta är små, mycket små,
mycket små."

(efter Wolgers/Adolphson)

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LIST OF PAPERS

The thesis is based on the following papers referred to in the text by their Roman numerals:

- I Tufvesson B, Johnsson T: Occurrence of reo-like viruses in young children with acute gastroenteritis. *Acta path. microbiol. scand. Sect B* 84:22-28, 1976.
- II Tufvesson B, Johnsson T: Immuno-electro-osmophoresis for detection of reo-like virus: Methodology and comparison with electron microscopy. *Acta path. microbiol. scand. Sect B* 84:225-28, 1976.
- III Tufvesson B, Johnsson T, Persson B: Family infections by reo-like virus. *Scand. J. Infect. Dis.* 9:257-61, 1977.
- IV Stintzing G, Bäck E, Tufvesson B, Johnsson T, Wadström T, Habte D: Seasonal fluctuations in the occurrence of enterotoxigenic bacteria and rotavirus in paediatric diarrhea in Addis Ababa. *Bull WHO* 59:67-73, 1981.
- V Tufvesson B: Detection of a human rotavirus strain different from types 1 and 2 - a new subgroup? *Epidemiology of subgroups in a Swedish and an Ethiopian community. J. Med. Vir.* 12:111-17, 1983.
- VI Tufvesson B, Polberger S, Svanberg L, Sveger T: A prospective study of rotavirus infections in neonatal and maternity wards. *Acta Paediatr. Scand.* Submitted.

INTRODUCTION

Although gastroenteritis and its consequences are probably one of the major causes of morbidity and mortality among children all over the world, the investigation of viral gastroenteritis had a late start.

It has been estimated that in 1975 approximately 500 million episodes of diarrhea occurred among children under the age of 5 in Africa, Asia and Latin America, resulting in 5 to 18 million deaths (35). At the beginning of that decade known bacterial and viral pathogens accounted, however, for less than one third of the cases (36,65), whereas two thirds of all diarrheal episodes were of unknown etiology. Until 1972 there was little evidence that viruses were of any importance for diarrheal disease (152). In that year, however, immunoelectron microscopy was used on stool samples from children suffering from Norwalk agent diarrhea, which made it possible to identify viral particles in the samples (84).

In 1973 another virus was identified in duodenal biopsies and stools from children with non-bacterial gastroenteritis, using direct electron microscopy (EM) (14,54). This new virus was initially given various names, such as duo-virus, reolike virus, infantile gastroenteritis virus, human reoviruslike agent (HRVLA) but rotavirus, the name proposed by Flewett et al (56), has now become the official generic name (112). The virus has been found to be a major cause of diarrhea among children all over the world (152).

With the introduction of EM for direct screening of faecal samples other viruses have also been detected in stools from children with acute gastroenteritis, including astrovirus, calicivirus, coronavirus, minirovirus and non-cultivable adenovirus (33,57,101,107,108,148).

Thus, acute viral gastroenteritis is now recognized as a common illness that affects all age groups and occurs in both epidemic and endemic forms.

The present study will deal mainly with rotavirus and its importance for acute gastroenteritis, especially in children.

THE VIRUS

A. Classification

Rotavirus has been classified as a separate genus within the family Reoviridae of the RNA-viruses (112), according to Table 1.

<u>Genus</u>	<u>Host</u>	<u>Representative disease</u>
reovirus	vertebrates	
orbivirus	vertebrates and insects	Colorado tick fever
rotavirus	mammals	gastroenteritis
phytoreovirus	plants	rice dwarf
Fijivirus	plants	maize rough dwarf
cytoplasmic polyhedrosis virus	insects	polyhedrosis

Table 1. Classification of the family Reoviridae

B. Morphology and physical properties

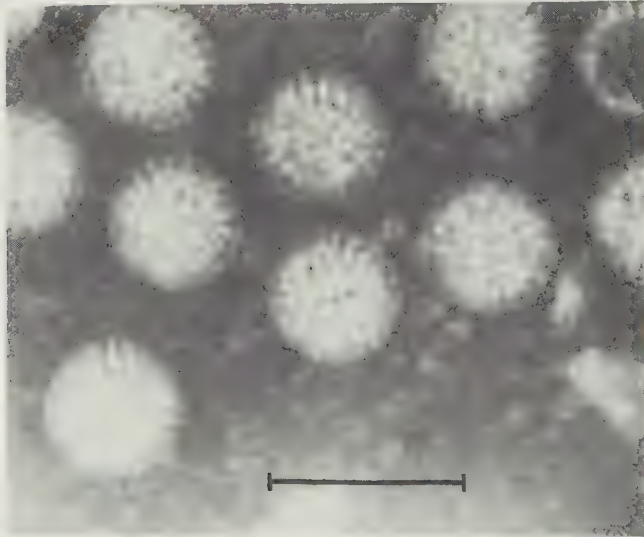


Fig 1. Rotavirus purified from a faecal sample of a child, 18 months old.

Bar represents 100 nm.

As seen by EM (Fig 1) the complete virion appears spherical with a smooth outline but the inner capsid has a subunit structure indicating icosahedral symmetry (55). When studied by scanning electron microscopy, freeze-drying technique, the architecture of the virus suggests an inner capsid of 132 capsomers with a skewed and icosahedral pattern. The outer layer was depicted as a shell with an estimated thickness of 5 nm, perforated by holes ($\emptyset$ 3 nm) regularly organized around five- and sixfold axes (131). The rotavirus appears in two forms in faecal samples (55). The complete form is about 70 nm in diameter.

The incomplete form is somewhat smaller, diameter 65 nm, and has a rough outline, having lost its outer shell. Empty shells are frequently seen in faecal samples (158). Tubular structures, obviously representing deranged rotaviruses, occasionally appear in samples taken late in the illness or during early convalescence (55,94). A summary of some important properties of the virus is given in Table 2.

<u>Properties</u>	<u>complete</u>	<u>incomplete</u>	<u>tubular</u>	<u>reference</u>
outline by electron microscopy	smooth	rough		55, 175
size (nm)	Ø 70	Ø 65	70x1000	55, 94
capsid	double shelled	single shelled		15
infectivity	+	-		49
density in CsCl (g/cm ³)	1.36	1.38		25
nucleic acid	double stranded RNA, 11 segmented			81
location of:				
neutralizing antigens	+	-		90, 181
hemagglutinating antigens	+	-		95
common antigen		+		56, 69
subgroup specific antigens		+		23

Table 2. Properties of the different forms of rotavirus particles.

C. Host range

The rotavirus particles found in the stools from infected children are morphologically identical to virusparticles found in the stools from the infected young of many species. There are, however, antigenic differences between different species, which explain the observed species specificity. These antigens have been demonstrated in the outer capsid layer of the virus (56,161). Rotaviruses have been identified from mice, calves, pigs, sheep, horses, monkeys, rabbits, deer and antelope among others (28,51,103,110,113,115,167,175). As indicated by the detection of rotavirus antibodies the virus also appears among dogs, guinea pigs and goats (166, 175).

Cross species transmission of virus has been reported, i.e. human virus has been experimentally transmitted to calves (105,116,117) piglets (24), monkeys



(179), lambs (143) and dogs (166). Piglets have also been experimentally infected with calf and simian rotavirus (72,168). Naturally occurring cross infections have not, however, been reported, except for a possibility of a bovine rotavirus in an outbreak of gastroenteritis among piglets (176).

D. Geographical range

Human rotaviruses appear to have a worldwide distribution, as measured either by rotavirus excretion and/or the antibody status in the population (1,5,6,9, 11,12,14,19,22,37,47,50,52,54,85,96,100,102,111,119,139,140,149,153,158,159, 192,194,I). Not even isolated populations, e.g. aborigines in Australia, are completely without antibody against rotavirus (63).

E. Clinical features and pathogenesis

The infection is usually transmitted by the faecal-oral route. The illness is characterized by the abrupt onset of vomiting and diarrhea after a short incubation period extending from 24 to 48 hours. Vomiting often precedes the diarrhea, which is of watery character and usually less than 10 stools each day. Pyrexia is a common feature. Mild to moderate dehydration is frequently seen. The illness is mostly selflimiting, but occasionally rotavirus infections are associated with more severe illness, and fatal cases have been reported (46,126,128,142,152,157).

Respiratory involvement has been described. Red throat, cough, otitis media were the most frequent symptoms. This was statistically more often associated to diarrhea attributed to rotavirus than diarrhea attributed to other pathogens. No virus excretion could, however, be detected in throat swabs (64,104). Further evidence of a transmission of rotavirus by the respiratory route appeared recently, when rotavirus was isolated from respiratory secretions in 8 % of pneumonia cases among children aged less than five years (136).

The first information on the pathological effects of the virus appeared already in the initial report about the virus (14), where duodenal biopsies were shown to have a patchy irregularity of the mucosal surface. Shortening and blunting of the villi could be seen by light microscopy. Virus particles could be demonstrated in the cisternae of the endoplasmic reticulum of epithelial cells by EM. It was also found that disaccharidases were decreased in the epithelial cells.

On examination of gastric and rectal biopsies from infected individuals the

histological findings were normal (119).

Studies of duodenal biopsies performed by immunofluorescence revealed rotavirus-antigen only in the cytoplasm of villous epithelial cells, while the cells of the crypts and lamina propria were spared. This involvement was also patchy, with some areas of mucosa spared. These findings had disappeared at control biopsies after four weeks. Corresponding studies from autopsy material showed that viral antigen was only present in the duodenum and upper jejunum. with no reaction in the stomach, colon, rectum or mesenteric nodes (42,119).

Animal models from calves, pigs, lambs and monkeys (43,117,144,179) have confirmed the hypothesis that mature enterocytes of intestinal villi are infected, followed by shedding of infected cells from the villi and the replacement by immature cells migrating from the crypts. The destruction of mature enterocytes result among other things in reduced levels of disaccharidases and a decrease in the absorptive surface of the intestine. Postinfectious intolerance of disaccharides, xylose and lactose is a frequent phenomenon (78), probably caused by the extensive destruction of enterocytes in the proximal intestine. Up to 55 % of patients with rotavirus gastroenteritis had abnormal lactose breath hydrogen tests, lasting 10-14 days after the onset of illness. Increase in diarrhea following lactose challenge was a rule among these patients. Lactase is possibly also the receptor and uncoating enzyme of rotaviruses (77). Patients with cystic fibrosis, and thus a drastic reduction of pancreatic enzyme levels, had antibody levels similar to those of normal subjects, indicating normal frequencies of infection and thus that pancreatic enzymes has little effect on the infectivity of rotaviruses (39).

The diarrhea caused by rotavirus is thus probably caused by decreased absorption, possibly with an element of osmotic diarrhea.

F. Detection of rotavirus/rotavirus antibody in conditions other than acute gastroenteritis.

A virus with the morphological and antigenic properties of a rotavirus has been found in filtrates from resected intestine of patients with Crohn's disease (173,174). However, it has not been possible to demonstrate any difference in antirotavirus-antibody levels between Crohn's disease/ulcerative colitis/controls (70). Thus, it is unlikely that rotavirus plays a primary etiological role in this disorder, but an acute attack of rotavirus gastroenteritis in pre-disposed individuals may play a precipitating role.

Konno et al (99) found rotavirus in 40 % of samples from patients with intussusception, while adenovirus could be detected in 27 %, which indicates that rotavirus could contribute significantly to this disorder. In later investigations adenovirus has, however, been found more frequently than rotavirus among infants with intussusception (122,125).

CNS involvement as a complication to rotavirus enteritis has been described in two cases (135) with encephalitis. One child eventually developed a fatal Reyes syndrome. In both cases rotavirus could be isolated from faecal samples, and in one case a seroconversion was demonstrated.

The frequency of rotavirus infections and other gastrointestinal pathogens has also been studied among bone marrow transplant recipients (190). Rotavirus and/or adenovirus were detected in 20 out of 78 patients with gastrointestinal symptoms. The mortality rate of patients infected with identifiable gastrointestinal pathogens was approximately four times that of the uninfected patients, although only 6 out of 17 deaths in the infected group had adeno- or rotavirus in the stools less than 11 days before death.

It is important to remember, however, that a virus that infects virtually every child can have a chance association with almost any symptom or syndrome!

THE AIMS OF THE STUDY:

- 1) to evaluate the importance of rotavirus as an aetiological agent in acute nonbacterial gastroenteritis in Sweden
- 2) to develop and evaluate different diagnostic methods for virus detection and screening of antibody response
- 3) to study the epidemiology of different subgroups
- 4) to compare the rotavirus epidemiology in Sweden and in Ethiopia, two communities with differences in climate and social development
- 5) to study the importance of neonatal rotavirus infection.

OWN INVESTIGATIONS

I. Materials and Methods

A. Patients. Studies I and II comprised all children who were hospitalized at

the paediatric clinic, Malmö General Hospital, for acute gastroenteritis from November 1974 to January 1976. The total number of patients was 244. During the first part of the follow-up (study I), a control group of 29 patients with non-enteric diagnosis was included.

In study III, the index cases came from the routine testing of children admitted to hospital for diarrhea from December 1975 to May 1976. In 31 cases the closest family members were included in the study, and screened by faecal examination and serology. The total number included in this study was thus 31 index cases, 27 siblings and 59 adults.

In study IV, which was conducted for a year, from March 1977 to February 1978, at the Ethio-Swedish Paediatric Clinic (ESPC) Addis Ababa, Ethiopia, a total of 962 patients was included, and also a control group of 191 children. The controls were not perfectly age and sex matched.

Study V comprises faecal samples with a positive rotavirus identification from all the studies above plus routinely diagnosed patients from Malmö General Hospital from February 1976 to December 1981. We also included 139 samples from a study of children in Addis Ababa with severe diarrhea requiring rehydration during 1979 (160). The total number of subjects in the studies and their age distribution are summarized in Fig 2 and Tables 3 and 4.

Study VI comprises samples from 553 infants and 542 mothers collected from January to April 1983 in the maternity wards within 7 days after birth of the infant. Samples from 150 infants attending the neonatal unit from January to August 1983 were also included. Faecal specimens were taken from these infants once a week until their discharge from the hospital.

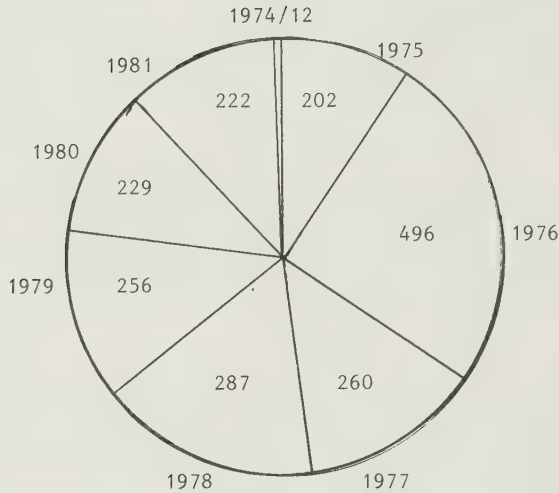


Fig 2. Number of gastroenteritis cases tested each year at Malmö General Hospital.

0 - 6 months	384
7 - 12 months	537
13 - 24 months	525
25 - 48 months	164
> 48 months	354

Table 3. Age distribution of patients with gastroenteritis at the paediatric clinic, Malmö General Hospital, during 1974-81.

	1977-78; outpatients	1979; hospitalized
0 - 6 months	288	65
7 - 12 months	289	89
13 - 24 months	242	18
25 - 48 months	95	
> 48 months	48	3

Table 4. Age distribution of patients with gastroenteritis in two materials from Addis Ababa.

B. Clinical observations. Diarrhea was defined as four or more loose mucoid or bloody stools, or more than one watery stool per day. Persistence of diarrheal stools was noted. In study I and IV the occurrence of vomiting, fever and dehydration was also registered. In case of dehydration, it was graded as mild (less than 5 %), moderate (5-10 %) or severe (> 10 %) weight loss according to clinical signs (26). In study VI, the clinical features mentioned above were recorded. Overtemperature, undertemperature, elevated levels of C-reactive protein or increased leucocyte particle count was recorded as other signs of infections. Antibiotic treatment was registered. Respiratory problems, such as neonatal disturbance of breathing and pneumonia, were recorded. Requirement of incubator case was also noted. Birth weight and gestational age were registered

Nosocomial infections were recorded in study I, i.e. children receiving treatment based on other diagnoses than gastroenteritis and who had fallen ill either after more than 48 hours in hospital, or within 24 hours after returning home.

C. Specimen handling

1. Faecal samples were collected on admission from all patients in study I to V. In study VI, faecal samples were obtained from the children within 7 days after birth, and at the same time from their mothers. Faecal samples from infants admitted to the neonatal unit of the department of paediatrics were taken once a week during admission.

At Malmö General Hospital, the samples were immediately sent to the Section of Clinical Virology, while the Ethiopian samples were kept frozen (-20°C) until sent to Sweden by air, a procedure normally scheduled to 5-7 days. At the laboratory, 20 % (w/v) solutions were prepared in a maintenance medium containing bovine serum albumin and antibiotics (penicillin 500 IU/ml, streptomycin 100 $\mu\text{g/ml}$, neomycin 100 $\mu\text{g/ml}$ and amphotericin B 4 $\mu\text{g/ml}$). After the initial testing these suspensions were frozen (-20°C) until needed.

2. Serum samples were obtained from patients in studies I and III. As a rule samples were collected 1-3 days after the onset of illness and also 2-3 weeks later. Paired sera were thus obtained from 85 patients and 23 controls in study I, and from 31 index cases, 8 siblings and 28 parents in study III.

D. Diagnostic procedures

D 1. Detection of virus

D 1. 1. Electron microscopy (EM)

EM was performed on all faecal samples in studies I, II, III and on 16 of the control samples in study IV. EM was also used on 76 faecal samples from study VI, where rotavirus had been detected by ELISA.

The screening procedure was performed, after an initial concentration by ultracentrifugation of the specimens. Faecal extracts were then negatively stained, and the virus findings were graded in a + to ++++ scale. The procedure has been described by Flewett et al (55).

Particle counts by EM was performed by comparison with a Latex suspension of known concentration (45).

D 1. 2. Immunoelectron microscopy (IEM)

In study I three paired sera from patients with gastroenteritis and rotavirus detectable in faecal samples were tested against a clarified human faecal extract containing rotavirus and against a suspension of bovine rotavirus (NCDV). Agglutination (%) was compared to agglutination of the same virus stock using a rabbit anti-NCDV-antiserum.

D 1. 3. Tissue culture

The faecal specimens from study I and III were cultured on green monkey kidney, vero and a local HeLa-like continuous cellline according to the routine technique of the laboratory. The NCDV used in studies I, II, III, IV was cultured on calf kidney cells using Eagles maintenance medium without serum. Occurrence of virus in the tissue culture lead to a second testing with EM according to D 1. 1.

D 1. 4. Immunoelectroosmophoresis (IEOP)

IEOP was performed according to Hansson et al (73) in 0.75 % agarose gels in barbital buffer (pH 8.6). Undiluted faecal extracts were placed in circular wells and tested against an equal amount of guinea pig antiserum, prepared from the corresponding bovine virus, NCDV. After completed electrophoresis, the plates were stained in Coomassie brilliant blue. This technique was applied to samples of studies II, III, IV and V.

D 1. 5. Enzyme linked immunosorbent assay (ELISA)

D 1. 5. 1. virus detection

ELISA was used retrospectively on all faecal samples collected for virus detection. The procedure, which is a modification of the ELISA described by Yolken et al (184) is summarized in Fig 3.

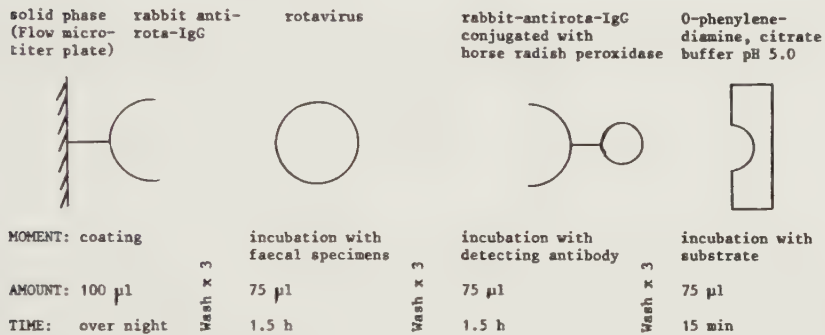


Fig 3. ELISA for detection of rotavirus.

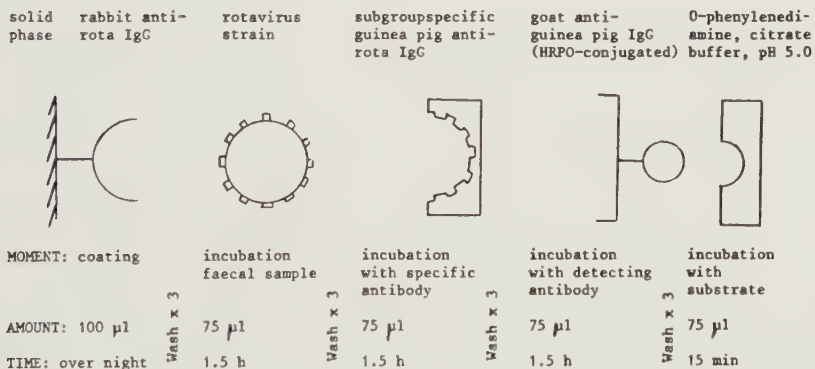


Fig 4. ELISA for detection of different subgroups of rotavirus.

When the test was completed, the plates were read in a Titertec Multiscan spectrophotometer at 492 nm. P/N values of ≥ 2.1 was considered as positive for rotavirus in the sample. A confirmatory blocking test was performed on positive samples (184).

D 1. 5. 2. subgrouping of rotaviruses

This test was used in study V, which also comprised positive samples from studies I-IV. The test is described in Fig 4.

Each strain of rotavirus was tested against three different subgroup specific antisera on the same microtiterplate. The plate was read spectrophotometrically, and the reaction was regarded as homologous if it exceeded the heterologous one with the same sample by at least 4 times.

D 2. Detection of antibody

D 2. 1. Complement fixation (CF)

A CF test, where NCDV-suspensions from tissue cultures were used as antigen after being clarified by centrifugation was developed according to the routine procedures of the laboratory (141). This test was applicated to sera from patients in studies I and III.

D 2. 2. Immuno-electroosmophoresis (IEOP)

Known rotavirus suspensions, purified according to a method described by Bishop et al (15) were used as antigens in the IEOP screening for antibody in paired sera obtained from study III. Twofold dilutions of the sera in saline were used. The procedure for the IEOP was the same as for antigen detection.

E. Other methods employed in the study

E 1. Crossed immunoelectrophoresis

10 μ l of each isolate of rotavirus was applied to 1 % agarose plates. Electrophoresis was run in the first dimension for 90 min with 10 V/cm. The second dimensional run was performed overnight at 2 V/cm into an antibody containing agarosegel. After the electrophoresis the plates were stained with Coomassie brilliant blue before examination. The scheme for this method has been described by Weeke (170).

E 2. Affinity chromatography for preparation of specific antisera

Guinea pigs were immunized with virus of known subgroups, kindly typed by dr G. Zissis, Bruxelles. The immunization scheme is described in study I. The hyperimmune sera were subjected to affinity chromatography, where rotavirus of a heterologous subgroup was coupled to CnBr-activated Sepharose 4 B gel (Pharmacia) according to a method described by Johansson et al (80).

RESULTS AND DISCUSSION

1. Evaluation of different methods for rotavirus detection

Rotavirus infections are almost always acute and particles are excreted in the faeces after the onset of illness. Most diagnostic procedures are therefore based on the direct demonstration of virus particles in faeces.

The tests used for rotavirus detection in the studies represent three different generations (Table 5).

The first generation, here represented by EM and IEM had the advantage over the others of demonstrating the virus (or viruses) visually. The most frequently employed variant is direct EM of negatively stained faecal samples after some form of concentration (4,15,54,119). The sensitivity may be increased by employing specific antibody to aggregate virus (= IEM). This makes the identification much simpler as the agent is present as complexes of antigen-antibody (4). The presence of artefacts is a complicating factor, especially when screening for small viruses. Although the techniques make it possible to detect virus in samples with an initial concentration of 10^6 particles/ml or more, it must be pointed out that the titre of biologically active virus in a specimen can be considerably less. Concentration and agglutination as per above are not obligatory for rotavirus detection by EM, as the virus excretion during the first days of illness is huge, estimated to 10^{13} particles/ml (152). EM still remains the reference method in laboratory diagnosis of rotavirus infections.

The second generation of tests had about the same sensitivity as the first, but the advantage of making it possible to process larger amounts of samples in a short time. It must be pointed out, however, that by relinquishing the first generation tests, one also loses the possibility of detecting more than one type of virus in each test system.

Generation	Representative tests	Means of detection	Sensitivity (lowest detectable amount of virus/ml)	+/-
I	EM IEM	} direct } visualization	10^7 $10^6-10^7(60, 132)^x$	+: can differentiate between two or more possible viruses in the sample -: time consuming, low sensitivity, expensive
II	IEOP CF IF	} antigen/antibody } reaction + sub- } sequent step for } visualization	$5 \cdot 10^7$ $10^7 (192)^x$ comparable to EM (182) ^x	+: can process several samples at one time, relatively fast, inexpensive -: requires specific antibody (= only one virus can be detected by each test), low sensitivity
III	ELISA RIA		10^5 better than EM (38, 121, 184) ^x	+: fast, high sensitivity, processing of large amounts of samples possible, inexpensive -: only one virus can be detected by the test, increased risk of false positives
IV	USERIA	- " -	100-1000x better (75) ^x than ELISA, RIA	

x) reference no.

Table 5. Some rotavirus tests, their means of virus detection, draw-backs/advantages and sensitivity

In our studies, the initial means of detecting rotavirus was by EM and IEM (I). Out of 114 children with gastroenteritis studied, 61 (54 %) were found to excrete virus, while a control group of 29 patients was negative. Study II comprised the samples tested by EM in study I, plus an additional 130 samples from new gastroenteritis patients. Here the ability to detect rotavirus by EM was compared to IEOP (Table 6).

Samples from	EM		IEOP		
	rota +	other viruses	neg	rota +	rota -
Patients with diarrhea (study I)	61	12	41	57	57
Controls (study I)	0	4	25	0	29
Fresh samples from patients with diarrhea	63	6	61	63	67

Table 6. EM and IEOP for detection of rotavirus in faecal samples.

Out of the positive samples from study I, 93 % were also positive by IEOP. The other part of the study, where double coded samples were tested first by IEOP and then by EM, gave a 100 % correlation between the two tests, both of them detecting 63 positive samples. Titration studies demonstrated that IEOP could detect virus concentrations of $5 \cdot 10^7$ particles/ml or more, compared to 10^7 particles/ml by EM.

The procedure we used for our IEOP was the same as described by Hansson et al (73) for the detection of HB_sAg. The electrophoresis was performed in a carefully selected batch of agarose gel. After electrophoresis the plates were stained with Coomassie brilliant blue before the final examination. The steps mentioned above were of great importance to the sensitivity of the tests, as a difference in sensitivity of 3-4 times depending on batch and manufacturer of agarose has been recorded (73). Furthermore, the staining procedure in our study meant an increase by at least 50 % of positive specimens.

Immunoelectrophoresis has also been used in two other studies for detection of rotavirus (120,146). Both find the technique highly specific but less sensitive than EM, both in clinical specimen and especially in end point titrations. Important parameters for the sensitivity were agarose batches and staining techniques, while no difference in sensitivity was seen when comparing anti-NCDV-antibody to anti-human rotavirus-antibody.

The third generation has in our studies been represented by ELISA. Dilution experiments and comparison to EM revealed a lowest sensitivity of 10^5 particles/ml, i.e. 100 times increased sensitivity compared to EM (165).

Our variant utilized the three layer technique with rabbit-anti-rotavirus-globulin both for coating and detecting antibody (peroxidase conjugated), which is the simplest and fastest way to detect virus. A similar technique is also used in commercial tests now available (Rotazyme - Abbott, Enzygnost-rotavirus - Behring) which, however, use antibody from different species in the two layers. The original ELISA of this type utilized antibody against a bovine rotavirus, Nebraska Calf diarrhea virus (NCDV), for the detection of human rotavirus (50). Yolken et al (183) claimed that antibody against NCDV failed to detect small amounts of human virus, possibly on the ground of the antigenic differences among the viruses, thus recommending antisera against human rotavirus. Sarkkinen (137) found, however, an equal sensitivity between tests using anti-NCDV and anti-human rotavirus. Using anti-NCDV antibody there was no difference in reactivity between rotavirus strains of subgroups 1 and 2 (provided from Malmö), which should be of advantage in routine diagnosis with the primary intention of detecting all subgroups of the virus in one test.

A four layer variant has also been proposed (184), where the anti-rotavirus reagent is not conjugated, and thus can be used in a high dilution, which together with the amplifying effect of an additional conjugated antibody allows easier visual identification, which would be the main advantage of this variant.

With the introduction of ELISA and equivalent methods controls of specificity have become increasingly important, as the methods are the most sensitive available for a virus that is not readily isolated in cell culture. Most laboratories including ours, use a blocking test originally described by Yolken et al (183), employing pre- and postinfection sera from experimentally infected animals.

Nonspecific reactions have for example been recorded from faecal samples containing anti-immunoglobulin antibodies, possibly analogous to IgM rheumatoid antiglobulins in some sera. These reactions can be identified by not being blocked in the test mentioned above or by reaction with the solid phase even if it is coated with nonimmune serum. Pre-treatment of the samples with N-acetylcysteine reduces this activity (189).

In spite of the vastly increased sensitivity of the third generation tests, the

efficiency of these in detecting rotavirus from faecal specimens is only marginally increased as measured by the excess number of cases detected. The net gain appears when dealing with samples taken more than one week after the onset of illness. In our material we have found an increase of positive cases by 24 % among such samples during one year. Among samples taken within the first week no significant difference was seen between ELISA and IEOP. Further new techniques with even higher sensitivity are, however, being developed. One example of these fourth generation tests is the enzymatic radioimmunoassay (75), which probably will find its main use in detecting the very low concentrations of viral agents in environmental samples. Another development is the polyacrylamide gel electrophoresis of rotavirus RNA in clarified faecal extracts (30, 81,82,127,138,155). The test has been used in direct rotavirus diagnostics, but although sensitive, it is more complex and laborious, and mainly useful in the research of different virus variants (6).

In this section, the efforts to adapt rotaviruses to growth in cell culture must also be discussed. Early, a simian (110) and some strains of bovine rotavirus (53,114) were cultured on primary or secondary cultures of kidney cells with a visible cytopathic effect. Isolation of bovine rotavirus from faecal samples in the presence of trypsin has also been performed on green monkey kidney cells (8).

In contrast, cultivation of human rotaviruses presented greater difficulties initially. In the first report, human embryonic intestinal organ-cultures were used for the cultivation (177). After this isolation one isolate could be adapted to primary HEK cells (178). Growth of human rotavirus was also detected in pig kidney and bovine kidney cultures (3,10,29). These studies all report the use of immunofluorescence for the detection of virus, as no cytopathic effect was visible. With most faecal isolates a limited proportion of cells were infected initially and, with a decreasing proportion at each passage, the strain was lost after a few passages. The proportion of cells infected initially could, however, be increased by centrifugation of virus onto monolayers of cells (10).

A major advance was the adaptation of human rotavirus strains of different subgroups to grow in MA 104-cells after serial passages through gnotobiotic piglets (180). The best indication of virus growth was still immunofluorescence, but also a cytopathic effect was described.

Recently there have been reports of the successful cultivation of human rotavirus directly from faecal samples in African green monkey kidney cells (CV-1) and MA 104 cells (2,13) with a characteristic CPE. In CV-1 cells the CPE consisted mainly of increasing granularity and partial detachment from the glass, while in MA 104 increased granularity, rounding into clusters and ultimate sloughing of infected cells were reported. The CPE was evident after 6-9 serial passages. Rotaviruses with RNA-profiles corresponding to two different subgroups could be cultivated, but in both studies only one third of the attempted isolations were successful.

It must therefore be emphasized that the attempts to adapt human rotavirus to growth in cell culture are most important as steps in the progress of research, where the availability of large quantities of purified virus will simplify the production of specific hyperimmune sera and monoclonal antibodies, thus making more specific antigen analyses possible. Cultivable strains will also be of potential use in the production of a future vaccine. As a direct diagnostic tool the virus isolation is of no importance as other methods currently used are both simpler and faster to perform, cheaper and of higher sensitivity.

2. Different tests for detection of antibody to rotavirus (I, III)

Immunoelectron microscopy (IEM) has been employed mainly for the detection of antigenic similarities or differences between viruses of different species (56, 175), but can also be of use in detecting seroconversion among patients with acute gastroenteritis caused by rotavirus (57,86). In these studies antibody was seen in detectable amounts from a week after the onset of illness, with increasing amounts during the following weeks. The reading was usually performed as an estimation of the agglutination of virus caused by the antibody present.

In a pilot study (I), IEM was performed on three paired sera from patients with rotavirus excretion. Differences in grade of agglutination between acute and convalescent phase sera were detected in all three cases. In fact, no acute phase serum could agglutinate any virus particles, neither was any antibody visible on the surface of the virions. Using the convalescent phase sera, the rotaviruses were heavily coated with antibody and agglutinated, both when a human purified virus and when a bovine rotavirus (NCDV) was used as antigen. This was also considered as a demonstration of the close relationship between viruses of these two species and was utilized in the development of a CF-reaction.

Tissue culture grown bovine virus (NCDV) was purified and used as antigen in a CF-test for antibody screening, as preparations of human virus were shown to be anticomplementary when used in the test. This was applied to 108 paired sera (I), with the results shown in Table 7.

	subjects	significant rise in titre	antibody detected without rise in titre	no antibody detected
Rotavirus positive gastroenteritis	49	47	2	0
Rotavirus negative gastroenteritis	28	4	6	18
Gastroenteritis, faecal samples not tested	8	5	0	3
Controls	23	0	11	12

Table 7. Antirotavirus antibody in gastroenteritis

Among the cases of rotavirus positive gastroenteritis, 96 % also had a significant rise in titre against the NCDV, while no significant rises were seen among the controls. Antibodies as a sign of previous contact with rotavirus antigen were detected among 48 % of the controls.

The CF test was also compared to IEOP for detection of antibody (III), which revealed an equal sensitivity between the two tests when comparing antibody titres from 64 paired sera. However, an overlap was present among patients with significant rise in titre as seen in Table 8.

	Subjects	Significant rise in titre by		
		CF	IEOP	Total
Index cases	29	24	23	27
Siblings	8	2	3	3
Parents	28	12	13	15

Table 8. CF/IEOP for detection of antirotavirus antibody

Three index cases had a significant rise in titre by IEOP only and 4 by CF only. Similarly, one of the siblings and 3 of the parents had a rise by IEOP only, while 2 parents had a significant rise in titre by CF only.

The net gain with antibody screening is obviously in cases with adult patients, where virus excretion is sparse and of short duration. We could find serological evidence of infection in 54 % of adult contacts with or without symptoms of gastroenteritis.

The IEM initially used for serodiagnosis was rapidly replaced by CF, which until now has been the most common test for screening of populations and serodiagnosis of rotavirus infections (20,63,71,76,87,134). Initially the antigen employed was human virus from stools, but this was replaced by cell culture grown bovine virus (86,87,1). A comparison has shown that human rotavirus antigen gave the highest sensitivity in the test, followed by simian and bovine virus (89).

Indirect immunofluorescence with tissue sections infected by human or bovine rotavirus has also been used for both detection of IgG and IgM (20,42,48,88,196). The technique is considered more sensitive than CF but more time consuming. Enzyme linked immunosorbent assay (ELISA) and radioimmuno assay (RIA) for antibody assay are now frequently used, combining high sensitivity with high specificity, i.e. suitable for detection not only of IgG and IgM, but also of IgA in serum, breast milk and intestinal fluid (7,44,63,67,169,185,186,187). The ELISA is stated to be 16 times more sensitive than CF in detecting serum antibody (63).

Anti-rotavirus-IgM can be detected by ELISA from the 5th day after the onset of illness, and this approach means a possibility to detect infections by taking only one serum sample (185).

While antigen from different species can be used for CF, i.e. human, simian and bovine rotavirus, only purified human rotavirus is suitable as antigen in the ELISA-test due to a high nonspecific reactivity among negative sera especially against cell cultured bovine virus. This is possibly due to contamination from the cells with bovine antigens, to which many humans have antibodies (185).

Studies on the prevalence of antibodies to rotavirus have been performed both with CF and ELISA, indicating rapid acquisition of serum antibody during the age of 6-24 months, although by CF a lower percentage of the population have anti-rotavirus antibody. By ELISA the figures approach 100 % at two years of age compared to 50-70 % by CF using bovine virus as antigen (20,63,88).

In summary, detection of antirotavirus antibody is a diagnostic tool of value especially in cases with acute gastroenteritis among adults. As for the different methods, CF is the one to be recommended among those employed in the present study. This test is well-known and already exists in most laboratories, and can thus easily be adapted to detection of antirotavirus antibody, preferably using a bovine rotavirus as antigen. However, ELISA seems to be an even better choice, with the advantages of stable reagents, easy adaptability to different classes of antibody, single dilution tests of samples and thus the requirement of only small amounts of reagents. Purified human rotavirus from faecal samples should be used as antigen.

3. Occurrence of rotavirus in cases of acute gastroenteritis (I-V)

During the years of the rotavirus investigations at Malmö General Hospital (1974-81) 1,964 children with acute gastroenteritis were included. Rotavirus was detected in 727 cases (37 %). In study I, comprising samples from the six months of the year with the highest incidence of rotavirus infections, 54 % of the patients excreted rotavirus. Counting also serological evidence of rotavirus infections, 62 % of the gastroenteritis cases observed during the period were caused by rotavirus. This pinpoints rotavirus as one of the most important etiological agents of acute gastroenteritis during childhood. This view is supported by other long-term studies on the occurrence of rotavirus in temperate areas around the world, where up to 50 % of gastroenteritis cases were due to rotavirus as diagnosed by EM and/or serology (27,41,88,98).

Scandinavian studies from Norway (195), where rotavirus was detected by EM in 44 % of the gastroenteritis patients, and a long term RIA-study from Finland (171) with 49 % of the patients excreting rotavirus also agree. Considering the differences in techniques and their differing sensitivity (compare page 20) these results seem to be consistent.

A long-term study from a developing area (Ethiopia) of the etiology of diarrhea in childhood (IV) revealed rotavirus as causative agent in 266 out of 962 cases (28 %). The materials in comparable studies are smaller, but report 26 % rotavirus caused diarrhea in India, and 32 % in Costa Rica (109,111). Rotavirus is still the most important of the agents investigated, but causes a smaller proportion of the childhood diarrheas. This is probably due to the relatively higher frequency of enterotoxigenic enterobacteria (ETEB) in these areas (1,154, 160, IV). In our study, ETEB was detected in 12 % of the cases.

Infections can occur simultaneously with rotavirus and other pathogens, both viruses, i.e. adenovirus, enterovirus (I) and enteric bacteria (IV). In our material 8 double infections with rotavirus + adenovirus and 3 double infections with rotavirus + enterovirus were spotted (I,III). Infections with ETEC and rotavirus at the same time were found in 40 (4 %) of the Ethiopian samples (IV). Double infections with enteric bacteria were not seen in study I, but have been reported from Sweden (126) in 2 % and Finland 5 % (171) of the cases. Cultivable enterovirus and adenovirus were frequently isolated among patients and controls selected from study IV. Both were isolated in higher frequencies from the controls (154). It has been recognized that these viruses are isolated with almost equal frequency from asymptomatic children, especially in developing countries (151).

The duration of virus excretion was studied in 5 cases (I), two of which lasted 6 and two cases 8 days. The fifth case, with prolonged diarrhea, excreted virus for 14 days after the onset of illness. Virus excretion was in these cases correlated to the duration of diarrhea. In contrast, symptom free virus excretors were detected in 5-8 % of Ethiopian control patients (160,IV). This high rate reflects the difficulty of finding suitable control subjects without a known recent history of diarrhea in these areas. Other authors utilizing EM for virus detection found excretion lasting 3-11 days (57,97,119), with the exceptions of children with prolonged diarrhea where the excretion continued up to 23 days (57), and adults with shorter lasting excretion (22). Although using the more sensitive RIA, Vesikari et al (171) could detect virus excretion only until 7 days postinfection. In general comparable studies seem to agree with our findings that virus excretion correlates well to the episode of diarrhea in small children.

A survey of all rotavirus cases at Malmö General Hospital during 1974-81 revealed that specimens collected within 3 days after the onset of illness accounted for 448 (61.6 %) of the total positives, while specimens collected later than 7 days after onset accounted for only 65 (8.9 %). A maximum shedding of virus was detected on the third to fourth day after onset, as estimated by EM (I). With a Latex calibration method used in study II for particle count, the amount of virus in samples taken during maximum shedding was 10^9 - 10^{10} particles/ml faecal suspension. Virus concentrations in the stools of 10^{13} /ml during maximum shedding have been calculated by EM (60,152).

The data above show that the risk of spreading the infection generally lasts

as long as diarrhea persists. The maximal risk, considering the excretion of virus, would be 3-4 days postinfection.

As regards diagnosis of the infection, samples taken within 3-4 days are ideal, containing large amounts of virus. If this time limit is observed, the method chosen will be of little importance, as the methods currently employed are of high sensitivity.

4. Distribution of rotavirus infections according to season and age (I, III, IV)

The seasonal distribution implied in the early study (I), with a variation that coincides with climatological changes, a peak occurring during the coldest months every year, could be confirmed in a follow up of all 727 cases detected at Malmö General Hospital (Fig 5).

In Ethiopia, two peaks of rotavirus infections could be demonstrated during the year, the first during June (42.7 % of the patients positive) and the second during November (36.4 % positive). These peaks were not as distinct as those for other pathogens in that study e.g. ETEB. A definite correlation to the climatological factors studied could not be found, but one peak coincided with the period of the small rains. The monthly distribution of the agents studied is seen in Fig 6.

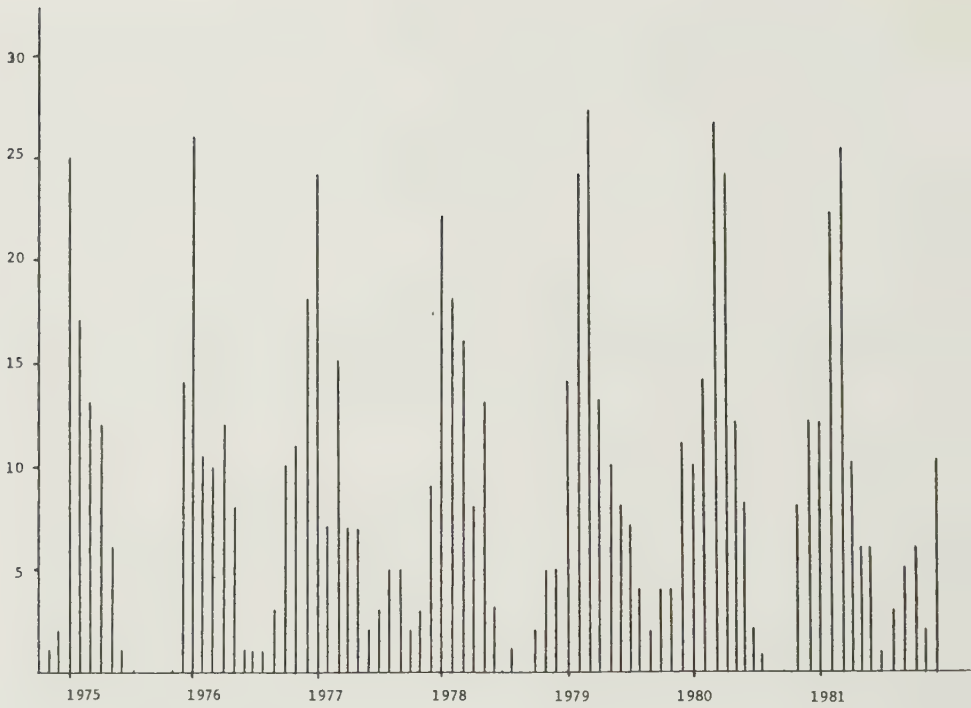


Fig 5. Monthly incidence of rotavirus infections (Malmö General Hospital 1974-81)

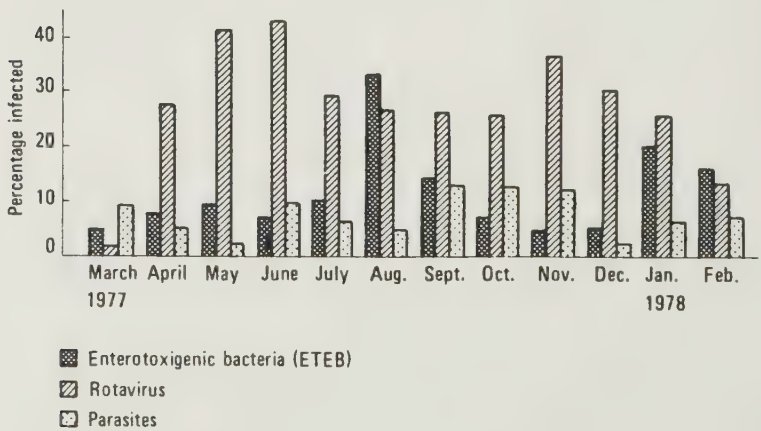


Fig 6. Frequency of isolation of enteropathogens by month (ESPC 1977-78)

Most reports from areas with temperate climates agree on the association between winter gastroenteritis and a peak prevalence of rotavirus infections (27, 41,88,98,171), while summer diarrhea is associated with rotavirus infections in 0-20 %. Where diarrhea in developing areas is concerned, reports claim that rotavirus is more common during the cooler months (109), the dry season (111) and the "small rains" (153). These studies are either of shorter duration than a year or comprises small numbers of patients. Thus, there is no definite evidence of a clear correlation to climatological factors. It is possible that rotavirus is a year-round pathogen in hot climates, without the seasonal variation seen in temperate areas. However, a nine month study from Kuwait (5) claims significant increase in rotavirus infections with decreasing temperature and increased humidity and rainfall.

In study I, rotavirus infections causing clinical illness were most commonly seen in the age group of 7-12 months, with an age range of 20 days - 4 years. The age distribution in the whole material confirms the existence of an age correlated peak of infections, although the peak interval is broadened to 7 - 24 months (Table 9).

In the age group 0-6 months no significant difference as regards frequency of positive samples was seen between patients belonging to the younger and older part of the group. The age range where virus excretion has been detected in association to clinical illness has extended to 5 days - 77 years, although the detection of rotavirus in faeces among elderly remains rare.

	Number of positive isolations	Frequency of positive samples in the group (%)
0- 6 months	131	34.1
7-12 months	248	46.2
13-24 months	248	47.2
25-48 months	56	34.1
> 48 months	42	11.1

Table 9. Age distribution among rotavirus excretors (Malmö 1974-81)

The relative frequency of rotavirus infections among Ethiopian children with gastroenteritis, who attended the outpatient department, ESPC, was lower than in Malmö in the groups less than 4 years old. The difference between the age groups was also less distinct (Table 10).

	Number of positive isolations	Frequency of positive samples in the group (%)
0- 6 months	77	26.7
7-12 months	90	31.1
13-24 months	71	29.3
25-48 months	22	23.2
> 48 months	6	12.5

Table 10. Age distribution among rotavirus excretors (ESPC 1977-78)

Among children hospitalized for severe diarrhea at ESPC 1979 a pattern more like the Swedish one could be seen. The frequency was highest among children of 7-12 months of age (45 %), coinciding with the weaning period (160). Most studies report a similar peak of incidence to that in Malmö among children 6 months to 2 years of age (27,41,88,98,119,171,195). In one study, however, 43 % of the infected children were less than 6 months old (142). Reports from developing countries state, however, a higher proportion of infections, up to 88 %, during the age of 2-12 months (5,133).

Studies on rotavirus infections, especially in humans, have mainly been concerned with the association to symptomatic disease, resulting in the above impression of a peak of infections in ages 7-24 months. However, recent reports on rotavirus infections in neonates, adolescents and adults have shown that asymptomatic or subclinical infections are common (18,22,76,106,157,164). Thus, an extended search for rotavirus in mild diarrheal disease among all ages should be important in order to explain the true pattern of this disease.

5. Rotavirus infections at obstetric and neonatal units (VI)

During a period of three months, rotavirus was identified in a total of 70 out of 553 (12.7 %) of the newborn within one week after delivery. Asymptomatic infections were responsible for 90 %. There was no difference in the monthly distribution of the infections. Only 7 out of 542 (1.3 %) of the mothers excreted rotavirus, all asymptomatic. In six of the cases infections among the mothers could be correlated to infection in the infants. In two wards with only obstetric patients, virus excretion was demonstrated in 11.6 % of the infants, as compared to 25.6 % in a mixed obstetric/gynecology ward.

In comparison, rotavirus excretion was demonstrated in 55 out of 150 (37 %) of the infants admitted to the neonatal unit of the paediatric clinic during eight

months in 1983, 85 % of which were asymptomatic infections. In 27 (49 %) of the rotavirus excreting infants at least the initial sample taken was negative. Hospitalization time was an important factor for the risk of developing the infection, as the frequency of positive cases increased from 21 % among those treated less than one week, to 70 % after more than 3 weeks treatment. As a consequence, a high proportion of prematurely born infants (30-36 gestational weeks) were found to excrete rotavirus. A low birth-weight ($< 2,500$ g) was also common among rotavirus excreters. Infected infants excreted rotavirus up to 6 weeks after admission. These patients showed a monthly distribution comparable to what is found among older children with symptomatic rotavirus infections.

Neonatal rotavirus infections were originally described by Chrystie et al (34), and have later been seen during longer periods in some major centres (17,18,29,31,32,35). Shorter outbreaks (16,46,123,164) have also been described, as well as failure to find any virus excreters among the neonates (150). This resulted in the opinion that neonatal rotavirus infection is endemic, with resident strains that differ from those present in the community (59,127). Most of the children excrete the virus asymptomatically between 5 and 10 days after birth (18,32,34). A lower incidence of excreters has been reported among breastfed compared to bottlefed children (34). In our study, there was a constant infection rate during the months at the obstetric wards, which together with the subgroup distribution (p.43) indicates endemicity of the infections. At the neonatal ward the subgroup distribution was similar to the obstetric wards. Furthermore, 49 % of the rotavirus positive infants were not excreting virus during the first week, which also indicated an endemic source. On the other hand, the high infection rate combined with the different monthly distribution at the neonatal ward, indicates a more complex situation with a possibility of also an outside source of infection.

A previous study of neonatal infection, could find no correlation of either low birth weight nor early gestational age to rotavirus excretion, in spite of a median duration of hospitalization of 3 weeks (32).

It has been said that the most important factor influencing the incidence of diarrhea is proximity to other newborns and handling of the infants by adults, who are not members of the family (17). This can be compared to the difference in infection rates in our study between obstetric wards and a mixed obstetric/gynecology ward. The mixed ward had more frequent visiting hours and probably also larger numbers of visitors, as there is a general prohibition of visits by

others than the close family at the obstetric wards. The same kind of argument can also explain the high frequency of infection at the neonatal ward, where the infants are nursed mainly by the staff.

The endemicity can be taken as a sign of how difficult rotaviruses are to eradicate once they have been established at a ward. It has been suggested, that the only way is to empty the ward, disinfect with formaldehyde fumigation and leave it for 7-10 days (17,59).

The importance of neonatal infection's lies in the risk of developing a symptomatic disease with a consequent prolonged hospitalization, which also would increase the cost of treatment. A weight loss has been described, both among the newborn with symptomatic and asymptomatic disease, which is important especially in developing countries where repeated episodes of diarrhea can cause weight losses that may impair the final growth of the patients (59). Furthermore, the infants are prone to spreading the illness to the close family, hospital staff and others as the children excrete large numbers of viral particles during a long period. On the other hand, neonatal rotavirus infections, although not conferring immunity against reinfection, protect against the development of a clinically severe form of the disease on reinfection in childhood (18).

6. Nosocomial infections (I)

Among the paediatric patients with rotavirus diarrhea, 33 % originally received treatment based on other diagnoses and were thus considered nosocomially infected (I).

Nosocomial infections have also been reported from other paediatric units, although with a lower incidence, 17-23 % (57,134). With the awareness of the problem of nosocomial infections, simple measures could effectively lower the rate. Isolation of infected children in separate rooms and simple hygiene precautions lowered the infection rate in our paediatric departments to approximately 10 % (165).

One study states the most likely mode of transmission to be indirectly from patient to patient on the hands of hospital personnel, finding actual infection or colonization of the staff a rare phenomenon (134). However, viral excretion has been detected both among nurses and medical students with diarrhea during the rotavirus season (22,93). Subclinical infections among adults are also common (93,III).

Nosocomial rotavirus diarrhea causes considerable morbidity and adds to the cost of hospitalization of the infants, with a reported hospitalization time for this illness of 1-15 days (p.38). Thus, proper isolation of children with diarrhea, including change of clothes and extensive handwashing among the nursing staff and visitors, seems to be worthwhile efforts. Alternative ways of managing diarrheal cases at home or in outpatient departments by use of oral fluid rehydration shortly after onset of diarrhea would also be of value (126, 134).

7. Sequential enteric illness due to rotavirus infections (V)

From 1977 to 1981, 18 cases of repeated rotavirus infection in children have been recorded at Malmö General Hospital. Faecal samples from both occasions were available for subgrouping from 10 cases. The initial infection occurred at 3-8 months of age, with the second at 11-34 months of age. Eight of the initial infections were of subgroup 1, while subgroup 2 and 3 caused one each. Subgroup 1 and 2 caused 1 and 9 of the secondary infections respectively. No patient shed virus of the same subgroup on both occasions.

The occurrence of sequential infections is interesting as it implies that a subgroup specific immunity is acquired at infections, while no child has shed the same virus on both occasions.

Sequential infections have been reported in two further studies, totally including 14 cases (62,188). All their primary infections were of subgroup 1, the second of subgroup 2 in 13 cases, while one had a repeated infection of subgroup 1.

The observation that infection with subgroup 1 does not induce cross-protection against subgroup 2 has also been reported after experimental infection of piglets with human rotavirus, and was probably due to a lower degree of viral replication and shorter duration of virus excretion. In consequence, rotavirus of subgroup 2 was also found more immunogenic than subgroup 1 (193).

The above observations are important as one of the current approaches to prevention of this illness involves the use of a vaccine which consequently must induce resistance to all existing subgroups to be effective.

8. Secondary infections in the family related to rotavirus infections among children (III)

Secondary cases of gastroenteritis occurred within 7 days after the onset of illness of the index case in 19 out of 31 families investigated (Table 11).

	siblings (< 12 years)	adults
gastroenteritis	11	19
no symptoms	16	40

Table 11. Secondary gastroenteritis cases in the family

Four (36 %) siblings with symptoms of gastroenteritis excreted rotavirus while an etiological diagnosis could not be established in the other seven cases. One child without symptoms also excreted virus, and another showed serological evidence of a subclinical infection.

Adults with gastroenteritis showed serological evidence of infection in 37 % of the cases, but without any detectable shedding of rotavirus. Among adults without symptoms 20 % had a significant rise in antirotavirus antibody.

In total, approximately half of the sero-responding adults had subclinical infections. This does not agree with Haug et al (76), who only found episodes of acute gastroenteritis among family contacts shedding virus and/or responding serologically. That study is also one of the few where rotavirus in faecal samples were discovered after the cessation of clinical symptoms of gastroenteritis. On the other hand, the only other study on rotavirus infections in adult contacts found subclinical infections in 88 % of the cases (93). It is obvious from these three studies, that there is a high rate of family infections in the community.

60 % of the parents with serological response had antibody detectable by CF/IEOP already in the acute phase serum compared to 25 % among the children. The mean antibody titer was also 2-4 times higher among adults than among children. This indicates a booster effect and thus previous contact with the antigen. It is in agreement with findings of a secondary type of immune response among adults, i.e. high IgG-titers while IgM was difficult to detect (76).

Only in a few cases in these studies was it possible to demonstrate virus ex-

cretion in faecal samples from adults. Accordingly a serologic diagnosis is often best, where adult rotavirus diarrhea is concerned.

The reinfections among adults may be due to the same or different subgroups as on the previous exposure. Our study only measured group specific antibodies, using antigens prepared from tissue cultures of bovine rotavirus, which made it impossible to study subgroups. However, examination of faecal samples from children with repeated infections indicates that reinfections are almost exclusively caused by another subgroup (p, 35).

Rotavirus infections in adults other than family members have been recorded in up to 40 % of staff members both at paediatric clinics and at other clinics (22). Outbreaks have also been reported among older school children, medical students, military personnel and tourists (21,73,93, 118).

Community epidemics of acute rotavirus gastroenteritis among adults have been reported from Sweden (106), with approximately 3,000 patients, and from China with 12,000 patients (159). Sanitary and epidemiological observations suggested a common waterborne infection in both epidemics.

Most reports describe the adult enteritis as a mild disease. This type of milder response is probably due to resistance acquired from a previous contact with the same agent. Adults with asymptomatic infections or only mild symptoms are thus an important factor for the spread of the virus, since infants and young children with primary infections are not often in contact with other children of the same age group.

9. Clinical observations (I, III, VI)

Some common features of rotavirus gastroenteritis have been described above (p. 10). Our observations among small children with acute rotavirus enteritis have been summarized and compared with other reports in Table 12.

Our clinical findings concur with studies on rotavirus diarrhea reported from other areas. Vomiting almost invariably accompanies the diarrhea, either as a short preceding episode or during the first days of diarrhea. Most studies report episodes of fever in more than 50 % of the cases.

	Sweden (126, I, III)	Great Britain (104)	Great Britain (160)	USA (128)	Kuwait (5, 92)	Japan (97)	Bangla Desh (133)	Ethiopia (160)
DIARRHEA								
duration (days)	1-14	1-15	1-8	1-9	1->14	-14	5-6	1->14
mean " -	4	3	4	5	3	NR	5.2	4
VOMITING (%)	84	69	100	96	76	79	92	NR
duration (days)	1-6	1-6	shortlived	NR	(1->14)	<1	NR	NR
mean " -	3	2	<1	2.6	2	NR	NR	NR
PYREXIA (%)	72	62	85	77	38	56	92	34
duration (days)	1-6	NR	1-3	NR	1->14	<1	NR	NR
mean " -	3	NR	NR	1.3	2	NR	NR	NR
DEHYDRATION (%)	45 (7*)	39	49	83	33	NR	55*	71 (17*)
RESP INFECTION (%)	21	66	33	26	28	NR	NR	NR
HOSP. TIME (days)	1-15 (mean 4)	NR	1-9	2-14 (mean 4)	mean 9	10-14	NR	NR
MORTALITY (%)	0	NR	NR	NR	NR	0.8	NR	3
Number of in- vestigated cases	70	150	27	72	42	110	12	47
* severe dehydration NR not reported								

Table 12. Occurrence of some important clinical features among children with rotavirus diarrhea. A comparison between reports from different areas.

The diarrhea rapidly leads to isotonic dehydration, which in most cases is mild, but severe dehydration occurs both in areas with good access to paediatric care (41,119,I) and especially in less developed areas, as shown in the studies from Ethiopia and Bangla Desh (133,154,160). The fatal cases reported are due to this dehydration.

Involvement of the respiratory tract has been recorded to a different extent by different authors. The most common feature was a history of cough, rhinitis or otitis before admission combined with a red throat on admission (104,128). In one study, respiratory symptoms had a significantly higher association to rotavirus induced gastroenteritis compared with other types of gastroenteritis (104).

The illness is described as self-limiting, provided that adequate rehydration therapy is carried out, either orally or parenterally (104, 126, 128, 157). The reports above, however, are all concerned with inpatients, with a reported hospitalization time of 1-15 days. There are no data concerning the clinically less severe and probably more common cases managed as outpatients.

In a recent study of a community outbreak of rotavirus gastroenteritis in China (159), where patients of ages 12 to 72 years were studied, most clinical observations were in accordance with the findings reported in small children with rotavirus gastroenteritis. The major differences reported were a high frequency of abdominal cramps (63 %), and a low frequency of fever (1 %). The virus causing this epidemic is claimed to be a novel rotavirus, mainly affecting adults. In a Swedish outbreak (106), the symptoms reported were diarrhea, vomiting, headache, muscular pain and fever, with a short duration (1-3 days), as compared to the Chinese study.

In contrast to what has been said above of older children, among newborns at the obstetric and neonatal units only 10 % and 15 % respectively of the rotavirus excreting infants had mild symptoms of gastroenteritis. A low birth-weight (< 2,500 g) was twice as common among infants excreting rotavirus. There was no difference between excretors/non-excretors regarding respiratory problems or other signs of infection (VI). Thus, in our study, neonatal rotavirus infections were mainly asymptomatic, and when symptomatic only mild diarrhea occurred.

One previous study states that 77 % of neonatal patients shedding rotavirus

were symptomatic (32). Infections in neonates can also cause severe illness with bloody diarrhea and abdominal distention (46). Other studies have, however, found neonatal rotavirus infection to be asymptomatic in a majority of the cases (17,18,34,123,124). In contrast to our study, one report shows that infants requiring special care in a neonatal unit were much more likely to develop symptomatic illness (17). Explanations to a milder course can be maternal antibodies crossing the placenta, thus conferring protection to the neonate. Colostrum also contains antibodies which may afford protection from severe rotavirus illness (79,162,187). Breast-feeding is also associated with decreased incidence of detectable rotavirus infection (35).

10. Subgroups and serotypes of rotavirus (V)

Two 'serotypes' have originally been described by Zissis et al (191), who used CF and antisera produced in guinea pigs for identification. Similar results have later been obtained using ELISA (129,188). Recent work has shown that these 'serotypes' should more properly be referred to as subgroups and that the term serotype should be reserved for the identification of antigens reacting with neutralizing antibodies consistent with the definition of serotypes among enteroviruses (90). Analysis of viral reassortants produced during coinfection with noncultivable human rotavirus and a mutant of cultivable bovine rotavirus indicated that these antigenic specificities were clearly dissociable and thus appeared to be coded for by different genes (68,181). The present definition of the subgroup specificity is based on ultrastructural differences in VP6, which is the translation product of the genomic RNA-segment 6 of the rotavirus. This is the major inner capsid protein, and contains both the antigenic region for the subgroup specificity and the common rotaviral antigen (69).

The two subgroups mentioned have been generally accepted as the predominating ones, generally using CF and ELISA as test methods (61,102,172,188,191). Except for the human rotavirus, subgroup 1 also includes the bovine rotavirus strain NCDV, the 0 agent, the simian SA11 virus and the porcine rotavirus as seen by immune adherence hemagglutination assay, while subgroup 2 was only found in human strains (90).

During our attempts to subgroup Swedish rotavirus strains we found six strains of a new variant different from the accepted two subgroups as seen by crossed immunoelectrophoresis (CIEP) (V). The test enabled us to demonstrate reactions between rotavirus antigens and antibody at two different positions in the gel,

representing the common antigen and a subgroup specific reaction. Three different patterns could be demonstrated, indicating 3 subgroups in our material. The new variant will thus in the following be called subgroup 3. This two peak pattern in CIEP has also been described by Grauballe et al (66). The existence of a third subgroup has also recently been suggested by Lambert et al (102).

The subgroups are of clinical and epidemiological importance, as sequential infections of different subgroups have been found in the same patient, suggesting the induction of only subgroup specific resistance upon infection (62,188, V). It must, however, be mentioned that also heterosubgroup serological response has been observed among adult volunteers, who had been given virus of subgroup 2 orally. All these showed signs of previous contact with rotavirus, with antibody in their prechallenge sera (91).

Rotavirus serotypes are designated as differences mainly in VP7, which is the major neutralizing antigen and the translation product of genomic segment 9. The antigen is located in the outer capsid (40). Four distinct serotypes of human rotavirus have been characterized by NT and plaque reduction assay (58, 68,156,163,181).

The serotype specific antigen may also be of importance in protective immunity (91).

Rotavirus strains are also distinguishable on the basis of molecular weight of their RNA segments. This test is performed by polyacrylamide gel electrophoresis. There are suggestions of at least 32 different electropherotypes of rotavirus (147). An association between the RNA pattern and the subgroup specificity has been observed, where RNA segments 10 and 11 from subgroup 1 were more slow-moving than those from subgroup 2 (83).

11. Epidemiology of different subgroups of rotavirus (V,VI)

Rotavirus positive samples collected from patients with acute gastroenteritis at Malmö General Hospital 1977-81 were subgrouped by ELISA. There was the same distribution for all years except for 1979, when the new subgroup appeared, as indicated in Table 13. Corresponding figures from Addis Ababa during three years, the first two years from the outpatient department and the last year from inpatients with severe diarrheal disease requiring parenteral nutrition, are also included.

	Malmö General Hospital			Addis Ababa		
	Subgroup 1	2	3	subgroup 1	2	3
1977	28	72	-	36	64	-
1978	27	73	-	35	65	-
1979	31	63	6	26	74	-
1980	24	76	-	not tested		
1981	23	77	-	not tested		

Table 13. Yearly distribution (%) of rotavirus subgroups among patients with acute gastroenteritis

Among children less than six months old there is no significant difference in subgroup distribution (1/3 type 1, 2/3 type 2) between Ethiopian outpatients, inpatients and Swedish inpatients. This distribution was maintained also among older children among Ethiopian outpatients, while in the other groups a shift to 1/4 type 1 and 3/4 type 2 could be seen.

The initial report on the distribution of rotavirus subgroups (188) suggested a fluctuation from year to year between the subgroups, but with a total of 3/4 of the strains of type 2 and 1/4 type 1. This variation was not present in our Swedish material. The difference could be due to the strict limitation in our material to hospitalized patients (= more pronounced illness). It is postulated that type 2 is more virulent than type 1 (188,193), indicating a large, stable proportion of type 2 in such a material. This also seems to be true for the inpatients at ESPC. A recent study over 45 months among Venezuelan children with rotavirus gastroenteritis confirms our findings of a stable distribution of the subgroups, finding a total of 85 % of subgroup 2 (172). In the outpatient study at ESPC also a continuous high proportion of subgroup 1 was recorded through the age groups, which seems consistent with their less serious symptoms.

The distribution month-by-month was not so stable. Clustering cases of one subgroup changed the distribution drastically, although both subgroups occurred together during the whole season. At Malmö General Hospital a detectable clustering of cases during September 1979 occurred at a paediatric ward. These cases could not be subgrouped originally by using the antisera corresponding to subgroup 1 and 2. Six out of nine patients were shown to be infected by the new variant of rotavirus (V). This variant has not been detected in any cases since then. The occurrence of this variant may be a transient phenomenon, but it was

originally detected by the use of antibody from commercial pooled human IgG, which should indicate a more common occurrence than our material suggests.

In a recent paper by Lambert et al a third subgroup which is as frequent as the others was described. They found that this subgroup arose from a division of subgroup 2. Evidence of this was found in the RNA-pattern of the strains (102). Antigenic drift among human rotaviruses during outbreaks of gastroenteritis has been demonstrated both by polyacrylamide electrophoresis and by hybridization tests (58,61,68). This indicates the possibility of detecting further subgroups of rotavirus, such as the new variant from Malmö.

Attempts were also made to determine the subgroup of samples from 70 virus excreting infants and 7 mothers at the obstetric wards as well as 55 virus excreting patients at the neonatal unit (VI). This was possible in a total of 41 (58.6 %) of the samples from infants and all samples from the mothers at the obstetric wards. 22 (53.7 %) samples from infants were of subgroup 1 and 19 (46.3 %) of subgroup 2, while all samples from the mothers were of subgroup 2. Differences in distribution between the obstetric and the mixed ward were observed. Subgroup determination was possible in a total of 29 (53 %) samples from the neonatal unit. The distribution was 14 (48.3 %) of subgroup 1 and 15 (51.7 %) of subgroup 2 (Table 14).

	subgroup 1	subgroup 2	subgroup not identified
obstetric ward I	11	8	14
II	10	3	11
mixed ward	1	6	4
neonatal unit	14	15	26

Table 14. Distribution of rotavirus subgroups at the maternity wards and the neonatal unit.

The above figures show a difference at the two obstetric wards and the neonatal unit compared to the distribution in the community, where 60-70 % of the cases are of subgroup 2 (V), and is in agreement with previous observations that strains resident in hospitals causing endemic infections and strains present in the community are different (59,127). Another study of subgroups in neonatal rotavirus infections states that all 20 cases tested were of subgroup 2, but with an electrophoretic pattern differing from strains among older children (18). In our study, the high proportion of strains of subgroup 1 may reflect an

endemic circulation at the wards of such strains. On the other hand, in the mixed ward with a high frequency of neonatal infections, the distribution of subgroups is similar to that found among older children with acute gastroenteritis. This can indicate, that a major part of the infections at this ward are not endemic. They may be a reflection of the differing spectrum of patients or of a larger number of visitors at the ward. A similar situation exists at the neonatal unit. Although the subgroup distribution and the delay of at least one week upon admission before turning rotavirus positive in 49 % of the cases (p.33) favours the hypothesis of an endemic infection, the monthly distribution indicates also an outside source of the infections. Thus, we find neonatal rotavirus infections mainly endemic in the obstetric wards, while in the mixed obstetric/gynecology ward and the neonatal unit endemic infections and outside sources of infection seem to be of equal importance.

To ascertain the endemicity, further investigations with polyacrylamide electrophoresis (127), as a possible way of detecting minor differences, will be needed.

Although the ELISA as a method is very sensitive, it is not the only test of choice for typing, where it has some drawbacks. Lambert et al (102) claims that CF is of higher sensitivity for typing and we have found that 15-20 % of samples found rotavirus positive by ELISA are not possible to subgroup (V). This may have more than one explanation: firstly the lower sensitivity of the typing-ELISA, secondly the possible existence of further subgroups which will not be detected, as the identification of subgroups is defined as greater reactivity with one reference serum than with others in this kind of test. At last, the repeated freezing and thawing that is common during the processing of the samples, reduces the reaction in the typing procedure.

Until now, the studies published have used subgroup antisera from guinea pigs produced by similar methods. Guinea pig antisera are considered the most specific for this purpose and have even been used to distinguish between bovine subgroups of rotavirus (145). However, White et al have reported an ELISA for subgrouping using monoclonal antibodies. This increases the sensitivity of the test, making it possible to subgroup 99 % of the samples (172).

CONCLUSIONS

The laboratory diagnosis of rotavirus infections can be established either by direct detection of virus from faecal samples or by serology. It is concluded that ELISA, as a relatively simple and highly sensitive test, is the method of choice, but almost any method for virus detection that is established at a laboratory can be used for screening faecal samples, as the virus excretion during the first days of illness is huge. Serological diagnosis can be established by methods currently used at the laboratories. The methods with the highest sensitivity demand the use of human virus as antigen. The less sensitive CF with NCDV as antigen, used in our studies, measures only antibodies to common rotavirus antigens, but is sufficient for clinical praxis. The diagnosis in children with diarrheal illness should be established by detection of viral antigen from faecal samples. In adult patients serology should be used, as the virus excretion in these cases is sparse and adults are easier to motivate for blood sampling.

Rotavirus is a typical winter pathogen in our area. During this period up to 2/3 of the cases of gastroenteritis investigated were caused by rotavirus. In contrast, no definite correlation could be found between the climatological factors studied and the two peak seasons in Ethiopia.

Rotavirus is an important agent of acute gastroenteritis among children in Sweden and Ethiopia, causing about 1/3 of the gastroenteritis cases investigated. In both areas it is mainly children between 7 and 24 months of age that are affected by the acute gastroenteritis caused by rotavirus. Asymptomatic or mild disease is common among neonates and adults. Secondary cases of gastroenteritis were detected in about 2/3 of the families of patients with acute rotavirus gastroenteritis. Half of the cases among the adults were subclinical infections. This group can be an important factor for the spreading of rotavirus infections.

Neonatal rotavirus infection is a common phenomenon in the maternity wards and the neonatal unit at Malmö General Hospital. At the maternity ward an average of every 8th infant and at the neonatal unit of every 3rd infant is infected. Infections are mainly asymptomatic, only 1/10 of the cases demonstrated mild symptoms of gastroenteritis. Infections are considered endemic in the obstetric wards with circulating strains especially of subgroup 1. In the mixed obstetric/gynecology ward and the neonatal unit a different subgroup distribution and a different monthly distribution, respectively, are indications of outside sources of infection which also are of importance.

Nosocomial infections are common. In the first season of our studies 1/3 of the patients originally received treatment based on other diagnoses.

Sequential illness due to rotavirus infections in children has been detected. The first infection, designated subgroup 1, occurred in these cases during the first six months and the second, of subgroup 2, at 1-3 years of age. The repeated infections can imply that only a subgroup specific immunity is acquired at infection or a possible lower immune response to infections of subgroup 1.

The diagnosis of rotavirus infection should be kept in mind especially when the patient is less than two years old, has a story of abrupt onset of vomiting and diarrhea with moderate fever and mild dehydration, often preceded by mild upper respiratory symptoms. In temperate areas the patients attend the clinic especially during the winter.

Rotavirus infections are selflimiting, but rehydration therapy is often needed. The diarrhea lasts for a median of 4 days, the vomiting for 3 days and the fever for 2 days. The incubation period is estimated to less than 48 hours. Virus excretion, and thus the risk of spreading the infection, is correlated to the duration of the diarrhea. The maximum shedding appears 3-4 days after the onset of illness.

At least two, possibly three, subgroups exist and typing of these by ELISA is of use in epidemiological studies, but the method is not suited to the discovery of new subgroups, as it relies on different reactivity between antigen and homologous respectively heterologous antibody. Other methods can help in this instance, eg. crossed immunoelectrophoresis. A tentative new subgroup, different from the two commonly recognized was detected in a cluster of cases from 1979. The importance of this variant is still uncertain, as only 6 cases have been identified so far.

The distribution of subgroups is stable between seasons among children with symptomatic rotavirus infections. Subgroup 2 predominates with 2/3 of the children less than six months of age and 3/4 among older children both in Sweden and Ethiopia. Subgroup 1 is of greater importance among Ethiopian outpatients older than 6 months, representing 1/3 of the cases.

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OCCURRENCE OF REO-LIKE VIRUSES IN YOUNG CHILDREN WITH ACUTE GASTROENTERITIS

*Diagnoses Established by Electron Microscopy and Complement Fixation,
Using the Reo-Like Calf Virus as Antigen*

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Tufvesson, B. & Johnson, T. Occurrence of reo-like viruses in young children with acute gastroenteritis. Diagnoses established by electron microscopy and complement fixation, using the reo-like calf virus as antigen. Acta path. microbiol. scand. Sect. B, 84: 22-28, 1976.

In the course of a six-month-study of acute gastroenteritis in children of ages up to six years, a reo-like virus was found in 54 per cent of the faecal specimens obtained at an early stage of the disease, using electron microscopy as screening test. By means of a concentrated complement fixation antigen, composed of a related calf diarrhoea virus cultivated in tissue culture, the rise in titre was found to be significant in 96 per cent of the patients whose faeces contained the reo-like virus. Antibodies were present in the remaining 4 per cent without rise in titre. In 10 per cent of the cases with gastroenteritis infection was caused by adenovirus or Salmonella. A probable aetiological agent was found in 71 per cent of the patients. It applies to 33 per cent of all cases caused by the reo-like virus that they were nosocomial infections.

Key words: Reo-like virus; gastroenteritis; complement fixation.

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Enteropathogenic bacteria, enterovirus and adenovirus are implied only in about 10-20 per cent of the cases of gastroenteritis in young children (4, 13). Until recently, the causative agent in the remaining cases has been unknown. The occurrence of reo-like virus in faeces and duodenal mucosa of patients with acute gastroenteritis has lately been described by *Bishop et al.* (2, 3). Findings of a similar virus in faecal samples from patients with acute gastroenteritis were at the same time reported by *Flewett et al.* (6, 7).

The incubation period of acute gastroenteritis caused by this virus has been estimated to range from 24 to 48 hours (4); excretion of virus will usually continue for one week after onset of illness (4, 7). Morphologically identical viruses have lately been reported by several investigators in other countries (1, 5, 9, 11, 13, 16). The virus reminds morphologically of the rcoviruses, but can be distinguished from these both serologically (7, 8, 9) and by its fine structure (2, 7, 8). It has been shown by immunoelectron microscopy (IEM) and complement fixation (CF) that

the Nebraska Calf Diarrhoea Virus (N.C.D.V.) and the virus of epizootic diarrhoea of infant mice (E.D.I.M.) serologically are related to the human virus (8, 9). So far electron microscopy (EM) has been the only method to identify the virus. The diameter of the virus is 67 nm. It consists of a core of 38 nm and two capsomer layers on top of each other (6, 7). *Flewett et al.* suggested to apply the term rotavirus to these reo-like virus, while *Bishop et al.* proposed duovirus (8, 4). Attempts have been made to culture the human virus, but so far, it has only been possible to make it grow out on organ cultures of foetal human intestinal cells (15). The calf virus, however, can be cultured on calf kidney cells (12, 14).

The aim of this work was to survey the incidence of acute gastroenteritis caused by this virus among children seen in the paediatric clinic of Malmö General Hospital. Furthermore, utilizing the serological relationship to N.C.D.V., the present authors intended to develop a CF-test to be used in diagnostics.

PATIENTS AND METHODS

Patients. During the period from November 1st 1974 to April 30th 1975, 114 children of ages up to six years were admitted to hospital on the indication of gastroenteritis; this group represents about half of the total number of patients in this age group with gastroenteritis admitted to Malmö General Hospital during this period. A control group of 29 children in the same age group admitted to the hospital during the same period but under other diagnoses, was included in the study, though the group was not directly matched to the gastroenteritis patients. The group of patients with gastroenteritis comprised 61 male and 53 female patients, the control group comprising 17 males and 12 females. It applies to both groups that the majority of patients were in the age group 6–18 months.

Faeces. Faecal samples were obtained from all patients immediately after admission to hospital, as a rule within one week after onset of illness, and from some patients also at later stages of the disease with a view to control the duration of virus excretion. Faecal extracts were negatively stained and examined by EM, as described by *Flewett et al.* (6, 7). Virus findings were graded according to a + to ++++ scale. All faecal samples were

also examined for *Salmonella* and *Shigella*, while no attempts were made to examine for enteropathogenic *E. coli*.

Sera. As a rule, sera were obtained from patients 1–3 days after onset of illness and also 2–3 weeks later. Paired sera were obtained from 85 patients with gastroenteritis and from 23 patients in the control group.

Tissue cultures. All faecal specimens were cultured on green monkey kidney cells, vero cells and on a local HeLa-like strain, according to the routine technique used in the laboratory. The calf virus was cultured on calf kidney cells using Eagles maintenance medium without foetal calf serum at a maximum of 15 days, the medium being changed every 5th day. The occurrence of virus was checked by EM as only a partial cytopathogenic effect was visible. Media and cell suspensions containing virus were pooled and stored at -20°C .

IEM. Three paired sera from patients with gastroenteritis and reo-like particles in faeces and a rabbit antiserum to the N.C.D.V. were tested against a clarified human faecal extract containing reo-like viruses and against a suspension of the corresponding calf virus according to a method described by *Flewett et al.* (8). The results were read as percentage of agglutinated particles.

CF-test. The Lincoln strain isolated by dr. C. A. Mebus, Department of Veterinary Science, University of Nebraska, USA, during an outbreak of N.C.D.V. was kindly supplied by dr. A. Meyling, Copenhagen, and used as CF-antigen. The pooled media were frozen and thawed three times. The fluid was clarified and virus was pelleted by centrifugation according to the same method as that used for faecal extracts. The pellet was re-suspended in 0.1 M veronal buffer, to one tenth of the original volume. The antigen had a titre of about 1/32 in chessboard titration and two units of antigen were used in the test. All paired sera were tested against the Lincoln antigen. Paired sera which failed to show a significant rise in titre against the Lincoln antigen were tested against adenovirus and enterovirus (Coxsackie B5).

RESULTS

Virological and serological findings. The result of the examination by EM is listed in Table 1. Reo-like virus was found in 61 out of the 114 cases (54 per cent) of gastroenteritis examined. The same test of all children in the control group was negative. Adenovirus and enterovirus occurred in 19 cases (17 per cent) in the gastroenteritis group and in 4 cases (14 per cent) in the control group. *Salmonella* was found in 3 cases (2

Table 1. *Enteric Pathogens in Faeces from Children with Acute Enteritis and from Children in a Control Group, Demonstrated by Electron Microscopy and Bacterial Cultivation*

Enteric pathogen	Children with acute enteritis (114)	Control children (29)
Reo-like virus	61 (54 %)	0
Adenovirus	15* (13 %)	1
Enterovirus	4§ (3 %)	3
Salmonella	3 (2 %)	0
Negative	38 (33 %)	25

* In four cases, adenovirus occurred together with reo-like virus.

§ In three cases, enterovirus occurred together with reo-like virus.

per cent) in the former group. In 7 cases (6 per cent), adenovirus or enterovirus was found together with reo-like virus. In 5 cases, in which the excretion of reo-like virus was followed in repeated samples, virus was demonstrable up to eight days after onset of illness, except in one case in which virus was excreted 14 days after onset of illness. There was a peak in excretion 3-4 days after onset of illness. No reo-like virus could be isolated in tissue culture, while adenovirus was isolated in 8 out of the 15 cases, in which adenovirus was found in faeces by EM. It applies to all four enterovirus that the findings by EM

could be confirmed by isolation in tissue culture.

The IEM revealed a close relationship between the calf virus and the human virus. As shown in Table 2, all human virus particles were agglutinated by human convalescent sera, while acute sera did not agglutinate the virus. The calf virus was agglutinated to about 80 per cent by the same human convalescent serum. The rabbit antiserum to N.C.D.V. agglutinated the human virus as well as the calf virus in 100 per cent.

Forty-nine paired sera were available from the 61 patients who by EM had been found to be positive. Forty-seven (96 per cent) showed a significant rise in CF-titre against the N.C.D.V. (Table 3), while antibodies were present in the two remaining paired sera without rise in titre. The first samples of serum were not drawn from these two patients until one week after onset of illness. Among the patients in whom EM failed to demonstrate reo-like virus in faeces a four-fold or greater response was detected in four patients. Another 5 cases were positive but samples of faeces were not obtained. In the control material comprising 23 paired sera there was no significant rise in titre.

In order to save sera, only a limited number of paired sera were tested in CF against adenovirus. One of the four patients with

TABLE 2. *Agglutination by Different Sera of Reo-like Virus from Man and Calf using IEM*

Patient	EM of faeces*	Agglutination (per cent)		
		Serum sample	Human virus	Calf virus
M.L.	+ + + +	acute conv. §	— 100	— 80
H.B.	+ +	acute conv. §	— 100	— 85
H.R.	+ + + +	acute conv. §	— 100	— 85
Rabbit 25 (inoculated with calf virus)	—	Hyperimmune serum†	~ 100	100

* findings of reo-like virus graded + to + + + +.

§ dilution 1/5.

† dilution 1/10.

TABLE 3. *Frequency of Complement Fixing Antibody against NCDV in Acute Gastroenteritis and in a Control Group*

	Number of paired sera tested	Significant rise in titre	Antibodies		
			No rise in titre	No antibodies	Not tested*
Patients with gastroenteritis, reo-like virus in faeces (EM)	49	47	2	0	12
Patients with gastroenteritis, no reo-like virus in faeces (EM)	28	4	6	18	17
Patients with gastroenteritis, samples of faeces not available	8	5	0	3	—
Control patients	23	0	11	12	6

* convalescent sera not available.

gastroenteritis, in whom both reo-like virus and adenovirus were found, showed a four-fold or greater antibody response, while 5 out of 9 patients with gastroenteritis in whom only adenovirus was present in faeces, presented a significant rise in titre against adenovirus. In 2 out of 17 patients with gastroenteritis by EM found to be negative and without significant rise in titre against the N.C.D.V., a fourfold antibody response against adenovirus could be demonstrated. No rise in titre against this antigen was observed in sera from patients in the control group. In one out of the four patients with gastroenteritis in whom enterovirus was found, a significant rise in titre against the Coxsackie B5-antigen was demonstrated.

In the series studied, acute gastroenteritis was most commonly seen in January (Fig. 1). Reo-like virus was the pathogen most commonly seen throughout the year. The total number of positive cases was highest in January, while the percentage increased during the Spring months. The number of cases in which adenovirus was demonstrable also reached a peak in January. The distribution of infections in relation to age of patients is illustrated in Fig. 2. Most of the patients with gastroenteritis belonged in the age group 7-12 months and the same applies to most cases in which gastroenteritis was associated with a presence of the reo-like virus. The youngest child found to be positive was 20

days old, the oldest was 4 years old. Occurrence of adenovirus showed the same age distribution as reo-like virus.

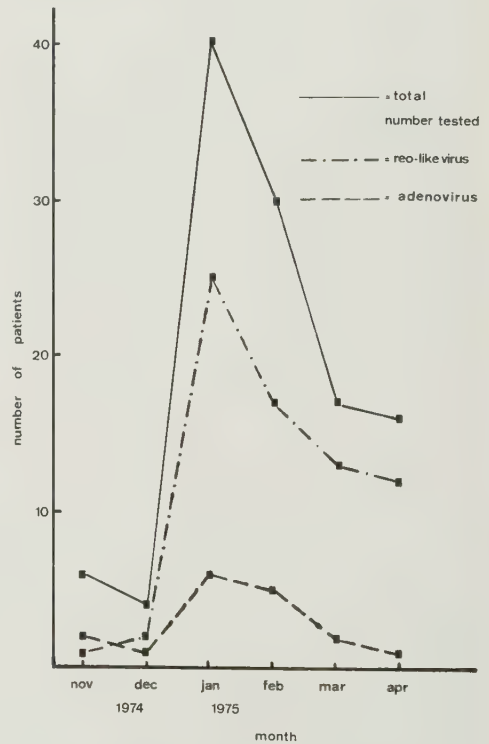


Fig. 1. Distribution over months of patients excreting reo-like virus and adenovirus and/or presenting significant rise in titre against these viruses in relation to total number tested.

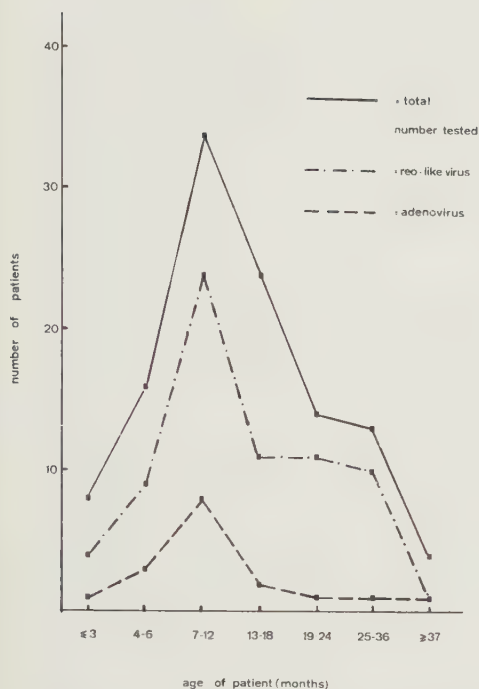


Fig. 2. Relation of reo-like virus and adenovirus excretion and/or significant rise in titre against these viruses to age of patients.

Twenty-two (33 per cent) of the patients with gastroenteritis and reo-like virus in faeces and/or a significant rise in titre against the N.C.D.V. originally received treatment based on diagnoses other than gastroenteritis. Gastroenteritis developed in these patients either when they had stayed in the childrens' ward for more than 48 hours, or within 24 hours after they had returned home. Fourteen (64 per cent) of these patients were less than one year old.

Clinical findings. The symptoms and their duration in 67 cases of gastroenteritis associated with the presence of the reo-like virus, diagnosed by EM and/or CF, are listed in Table 4. Diarrhoeas would usually persist for about 4 days. The temperature was elevated in 72 per cent of the patients, the fever lasting for 2-3 days and averaging 38°.7 C. Gastroenteritis did not run a fatal course in any case during the period of this study. One

TABLE 4. Clinical Findings in 67 Cases of Acute Gastroenteritis Caused by Reo-like Virus

	Number	%	Median duration, in days
Diarrhoea	67	100	4 (1-14)
Vomiting	56	84	3 (1-6)
Fever ($\geq 37^{\circ}.8$)*	48	72	2.3 (1-6)

* median temperature 38°.7 (37°.8-40°.2).

patient was moribund at the time of admission. A further four were dehydrated to such a degree that intravenous drop was required. In general, however, the patients were not severely ill.

DISCUSSION

Reo-like virus could be demonstrated by EM in 54 per cent of all cases of gastroenteritis studied, while this virus was not found in faeces from patients in the control group, thus indicating an association between the virus and the disease. The occurrence of virus in patients with gastroenteritis was most common during the colder season, a peak being seen in January. This, as well as the fact that virus is most commonly seen in children in the age group 7-18 months is in agreement with findings in previous studies (4, 7, 13). A further evidence of an association between the virus and the disease is the occurrence of increasing titre against the virus in paired sera. This was shown simultaneously by *Flewett et al.* (8) and *Kapikian et al.* (9) using IEM. The observations could be confirmed by the present authors in three paired sera from patients with gastroenteritis. *Kapikian et al.* (9) used faecal extracts as antigen in a CF-test and found a significant rise in titre in paired sera from EM-positive cases of gastroenteritis. By this method it was also possible to demonstrate the antigenic relation to the N.C.D.V. In the present study, where concentrated N.C.D.V. was used as antigen, a fourfold or greater serological response could be shown in 47 out of 49 (96 per cent) cases of gastroenteritis. The results obtained

TABLE 5. *Probable Aetiology in 114 Cases of Acute Enteritis in Young Children*

Reo-like virus	faeces positive (EM)	61	} (61.5 %)
	faeces negative (EM)		
	positive CF	4	
	faeces not available		
	positive CF	5	
Adenovirus	positive CF	7	(6.0 %)
Enterovirus	positive CF	1	(1.0 %)
Salmonella		3	(2.5 %)
Total positive		81	(71.0 %)
Negative		33	(29.0 %)

by the present authors by EM could be almost completely confirmed serologically by CF using a N.C.D.V.-antigen. This correlation between EM and CF is closer than that recently reported by *Kapikian et al.* (10), who only in about two thirds of the verified cases of gastroenteritis caused by reo-like virus could show rising titre against another strain of the N.C.D.V. In the present study, the four cases found to be positive by CF, but negative by EM, can be explained by the fact that the faecal samples from these patients were collected rather late in the course of the disease, more than one week after its onset. Taking into account the five cases from which faecal specimens were not obtained, the number of cases of gastroenteritis associated with reo-like virus totalled 70 out of 114 (62 per cent) in the present study (Table 5). Considering the incubation time, it is obvious that one third of the patients in whom the reo-like virus was the causative agent were contaminated during their stay at the hospital. If the 7 cases in which the titre against adenovirus increased, as well as the case involving enterovirus and the 3 cases involving *Salmonella* are included, a probable aetiology could be shown in 81 out of 114 cases (71 per cent).

EM is a very rapid and convenient method by which to demonstrate the reo-like particles, but an expensive equipment is required. In laboratories not equipped with an electron microscope the CF-test used in the present study is another possibility, even if the diag-

noses cannot be established in the early stage of the disease. CF can also be used for epidemiological studies. From a clinical point of view, it is urgently required to have the diagnosis established as the disease may be of severe nature. Thus, *Middleton et al.* (13) and *Bishop et al.* (4), have reported 7 and 5 fatal cases, respectively. In the present study, there were no fatal cases. Still, one patient was in very poor condition at the time of admission.

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IMMUNOELECTROOSMOPHORESIS FOR DETECTION OF REO-LIKE VIRUS: METHODOLOGY AND COMPARISON WITH ELECTRON MICROSCOPY

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Tufvesson, B. & Johnsson, T. Immunoelectroosmophoresis for detection of reo-like virus: Methodology and comparison with electron microscopy. Acta path. microbiol. scand. Sect. B, 84: 225-228, 1976.

In two separate studies, faecal samples were collected from children with acute gastroenteritis; the samples were tested both by electron microscopy and immunoelectroosmophoresis with a view to detect reo-like virus. In 94 per cent and 100 per cent of samples positive by electron microscopy in the first and second material, respectively, a precipitin line was found after electrophoresis, using a guinea pig antiserum against a purified bovine virus. No false positives were detected by this method.

Key words: Reo-like virus; gastroenteritis; immunoelectroosmophoresis.

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In small children, gastroenteritis associated with the presence of reo-like virus in faeces is now a well-known illness, which has been reported from various parts of the world (6, 9). The diagnostic method used in most trials reported is electron microscopy (EM) of faecal samples. However, this method requires expensive equipment and cannot be performed in all laboratories. A corresponding bovine virus, Nebraska Calf Diarrhoea Virus (NCDV), which can be cultured on calf kidney cells, was injected into guinea pigs by *Spence et al.* (8), and the resulting antiserum was used in complement fixation (CF) for direct detection of human virus. Culturing of human reo-like virus has until recently only been possible on organ cultures

of human intestinal cells (10). By now, however, *Banatvala et al.* have presented a system for *in vitro* detection of human reo-like virus, which involves culturing on pig kidney cells plus an additional immunofluorescent test (1). The NCDV has also been used as an antigen, surveying paired sera by CF (5, 9) and by immunofluorescence (11). The present work deals with immunoelectroosmophoresis (IEOP), a rapid and inexpensive alternative method for detection of human reo-like virus.

MATERIALS AND METHODS

Faeces: From a survey conducted November 1974-April 1975, 114 faecal extracts from children with gastroenteritis were obtained, as well as 29 samples from children with other types of illness. The oc-

currence of infection caused by reo-like virus in this study, carried out by EM and CF, has been presented in a previous paper (9). In a second study from May 1975–January 1976, the number of faecal samples from patients with gastroenteritis totalled 130. In the first study, the percentage of faeces in the extracts varied from a few per cent up to twenty per cent, due to shortage of material in the samples provided. In the second study, more faecal material was provided in each separate case and it was always possible to make 20 per cent (w/v) solutions.

EM: EM of the faecal samples was carried out according to the method described by *Flewett et al.* (3). Half of the squares on each grid were examined at a magnification of 22,500 $\times$. The number of virus particles in a purified antigen, as well as in some faecal extracts, was counted by comparison with a Latex suspension (particles $\varnothing$ 264 nm) of known concentration.

Antiserum: A bovine strain of reo-like virus (NCDV, strain Lincoln) was cultivated in calf kidney cultures. After purification of the virus by treatment with Arklone and polyethylene glycol, as described by *Bishop et al.* (2), guinea pigs were given intramuscular injections on days 1, 15 and 29. The injections contained a mixture of Freund's complete adjuvant and 25 μ g of the purified virus per animal. The animals were sacrificed on day 43. Double gel diffusion tests were performed using a micro-Ouchterlony technique, described by *Prince* (7), in order to check the identity of antibody with a number of paired human sera from EM-positive cases. Using faecal extracts as antigen, there was a sharp precipitin line against human convalescent sera, while there was no line between the antigen and the acute sera. Guinea pig antisera giving precipitin lines which showed identity with the convalescent sera were selected and pooled.

IEOP: The electrophoresis was performed ac-

cording to a method described by *Hansson & Johnsson* (4) in 0.75 per cent agarose (L'Industrie Biologique Francaise) in barbital buffer pH 8.6. Undiluted clarified faecal extracts were placed in circular wells and tested against an equal amount of undiluted guinea pig antiserum. Seventy samples could be tested on each plate. The electrophoresis was run for a period of 75 minutes at 7 V/cm. After electrophoresis, the plates were preliminarily read, submerged in saline overnight, stained with Coomassie brilliant blue and finally examined.

RESULTS

The results obtained in the first study are listed in Table 1. The number of positive samples after staining was 49 (81 per cent) of the 61 shown to be positive by EM. Eight of the remaining 12 which were positive by EM were also positive in IEOP after a ten-fold concentration in Lyphogel. Four samples which were negative by EM, but where corresponding paired sera showed significant rise in titre by CF, were also negative by IEOP. In tests of 82 faecal samples containing other viruses, samples from patients negative with respect to gastroenteritis, or samples from patients with other diseases, no precipitin lines were found. The results obtained in the second study in which all the faecal extracts contained 20 per cent faeces are shown in Table 2. In this study, all faecal samples were tested by IEOP, directly on their arrival, and all specimens were checked second-

TABLE 1. Comparison of EM and IEOP for Detection of Reo-like Virus in 143 Faecal Samples (December 1974–May 1975)

	EM result			Other diagnoses
	Reo-like virus	Gastroenteritis Other viruses	No virus*	
Total number tested	61	12	41	29
IEOP-positive before staining	6 (10%)	0	0	0
Additional IEOP-positive after staining	43 (71%)	0	0	0
Additional IEOP-positive after concentration	8 (13%)	0	0	N.T.
Total IEOP-positive	57 (94%)	0	0	0

* Corresponding paired sera from four patients showed significant rise in titre in CF.
N.T. = not tested.

TABLE 2. *Comparison of EM and IEOP for Detection of Reo-like Virus in 130 Faecal Samples from Patients with Gastroenteritis (May 1975–January 1976)*

	Reo-like virus	EM result	
		Other viruses	No virus
Total number tested	63	6	61
IEOP-positive before staining	31 (49%)	0	0
Additional IEOP-positive after staining	32 (51%)	0	0
Total IEOP-positive	63 (100%)	0	0

daily by EM, the IEOP result being unknown to the investigator. In this study, 31 samples (49 per cent) were found to be positive before staining and an additional 32 samples (51 per cent) were positive after staining, a total of 63 samples being tested, thus showing a 100 per cent correlation between IEOP and EM. No false positives were detected among 67 faecal samples from patients with other viruses or from patients negative with respect to gastroenteritis. Examination of precipitates from gel diffusion and IEOP experiments in EM, showed that they contained aggregates both of complete particles and particles lacking the outer capsid, as well as empty particles.

To estimate the amount of virus needed to form a visible precipitin line in gel, a titration of a purified antigen as well as clarified faecal extracts were tested by IEOP. A particle count was made on the same suspensions, using a Latex calibration method. The electrophoretic titre of the purified antigen was 1/128 after staining, while the clarified extracts gave titres of 1/64 and 1/16. There was a fourfold rise in all titrations after staining. The dilutions corresponded to about 5×10^7 particles/ml, which probably indicates the border line for a detection of reo-like virus by IEOP.

DISCUSSION

The IEOP method used in the present work was found to be as sensitive as EM, in contrast to findings in a preliminary study reported by *Spence et al.* (8), who out of 11

EM-positive faecal extracts found only 5 positive extracts by IEOP; if, however, a CF-test was used for identification of virus in faecal samples, 10 out of the 11 were positive. The present authors have tried to repeat this identification by CF, but the samples were often found to be anticomplementary. In the first study, where concentration of the faecal samples was necessary in 8 cases (13 per cent), the extracts often contained a lower percentage of faeces. These 8 samples were, according to the relative virus estimation in the former work (9), all + in the + to + + + + scale used. The low concentration of virus in many of the samples explains probably also the relatively low number of positive samples before staining in the first study. The suspensions used in the second study all contained 20 per cent faeces and with this concentration 49 per cent of the EM-positive were detected before staining, while all were positive after staining. The detection of virus by EM is, beyond doubt, a rapid test to be used whenever a diagnosis has to be established in the acute stage of illness. The performance of a number of tests, however, is time consuming and requires expensive equipment. The IEOP, on the other hand, is easily performed on a large scale, and it is extremely inexpensive as compared with the EM-method. Even in the case of a relatively small amount of virus in the sample, which would require washing overnight and staining to make a precipitate visible, the IEOP-method seems preferable.

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Family Infections by Reo-like Virus

Comparison of Antibody Titres by Complement Fixation and Immunelectroosmophoresis

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ABSTRACT. The families of 31 index cases infected by reo-like virus were examined for gastroenteritis symptoms, virus excretion and serological evidence of infection. Excretion of virus was seen only among the children in the families, and it occurred together with gastroenteritis symptoms in 97.2% of all cases. Virtually all infected children had a significant rise in titre in paired sera. Among the parents 25.0% had a significant rise in titre associated with infections with gastroenteritis symptoms and 28.6% with symptomfree infections. A comparison between complement fixation and immunelectroosmophoresis for detection of antibody showed that the tests gave similar results.

INTRODUCTION

Reo-like virus is a major cause of acute gastroenteritis in infants and young children from various parts of the world (4, 11, 17). Outbreaks of gastroenteritis associated with reo-like virus in nurseries are frequently described (2, 10, 13, 16, 20). The present authors found in an earlier study (17), that 30% of the gastroenteritis cases with infection by reo-like virus at a children's ward were nosocomial infections. It was therefore of interest to investigate the spread of virus in the family unit, a spread which was found to be typical among the enteric viruses already in the early fifties (8).

In most reports (1, 6, 9, 12, 14, 16, 17) the method used to demonstrate viral infections is electron microscopy (EM) on faecal specimens. In recent investigations Middleton et al. (12) and the present authors (18) have found immunelectroosmophoresis (IEOP) to be a rapid and sensitive method for direct detection of reo-like virus. Serological methods used for demonstration of antibody against reo-like virus are complement fixation (CF) and immunofluorescence (2, 3, 9, 14, 16, 17). In the present study a comparison has been made between CF, as introduced by us in an earlier study (17), and IEOP on acute and convalescent sera from the children and their parents.

PATIENTS AND METHODS

Patients. The group studied consisted of 31 families, in which one child, the index case, had been hospitalized under the diagnosis gastroenteritis acuta, with reo-like virus in the faecal specimen. Two faecal samples were collected from the parents and siblings of the index cases, the first immediately after virus excretion of the index case had been demonstrated and another 10 days later. From the 31 index cases, from 8 out of 27 siblings and from 28 out of 59 parents serum samples were drawn on the same occasions. 16 of the children were between 6 and 12 months old, and the remaining 42 between 1 and 12 years of age. The study took place during the months December 1975-May 1976.

Faecal suspensions. 20% (w/v) suspensions were made of the samples, as described earlier (18). The suspensions were homogenized, centrifuged at 5000 g for 30 min and tested. The samples were stored at -20°C.

EM. The screening was performed on all faecal specimens after concentration by ultracentrifugation of the material. The preparation and staining techniques have been described elsewhere (6, 17).

IEOP. A guinea-pig hyperimmune serum containing antibody against the corresponding bovine diarrhoea virus, Nebraska Calf Diarrhoea Virus (NCDV), was used in the IEOP screening of faecal samples, as described in previous works (7, 18). The IEOP antibody assay was performed in the same way with 2-fold dilutions of sera in saline and tested against a pool of human antigen, purified according to a method described by Bishop et al. (1).

CF. The test was performed with NCDV as antigen, as described previously (17). Four units of antigen were used.

Table I. *Distribution of gastroenteritis in 31 families with reo-like virus infections*

Symptom	Children (<12 yrs.)	Parents
Gastroenteritis	42	19
No symptoms	16	40
Gastroenteritis/ total no. tested	42/58 (72.4 %)	19/59 (32.2 %)

Tissue cultures. All faecal specimens were cultured according to the routine techniques of the laboratory (17).

RESULTS

Clinical findings

The 31 index cases all showed the clinical picture described for reo-like virus infections. One child among the index cases was severely ill and required parenteral nutrition during 5 days. In the families, secondary cases of gastroenteritis, among the children as well as the parents, occurred within 7 days after onset of illness of the index case. In 19 out of the 31 families studied at least one relative showed symptoms of gastroenteritis. Among the children and parents 72.4 % and 32.2 %, respectively, were taken ill (Table I).

Laboratory findings

Virus detection: The result of the screening of faecal samples which was performed by IEOP, and afterwards completed by EM and inoculation in tissue cultures, is presented in Table II. All 31 index cases were excreting virus in amounts measurable both by IEOP and EM. Among the siblings, 5 (18.5 %) were excreting the virus in faeces. One of them, 6 years of age, was shedding the virus in

extremely small amounts, which could be detected only by EM. The other four, all under 3 years, were shown to excrete virus both by IEOP and EM. Including the index cases, totally 36 out of 58 (62.1 %) children were excreting reo-like virus particles. The parents were all negative in the screening for reo-like virus of their faecal samples. Adenovirus infections were seen by EM in 6 cases among the children; 3 as double infections with reo-like virus among the index cases and 1 among the siblings. Two siblings and 2 parents were excreting only adenovirus. All viral particles seen were in the first collected faecal sample from each patient.

Serology. The results of the serological study within the families are summarized in Table III. Among the index cases, 27 out of 29 tested (93.1 %) showed a significant rise in titre either by CF or IEOP or both. Three had a significant titre rise only by IEOP and 4 only by CF. Two index cases had antibody by CF and/or IEOP without significant rise in titre. Two out of 8 siblings tested showed a significant rise in titre both by CF and IEOP, a further one only by IEOP. In total, 30 out of the 37 children tested (81.1 %) had a significant rise in titre in one or both tests. Out of the 28 parents tested, 10 had a significant rise in titre both by IEOP and CF, 3 only by IEOP and 2 only by CF. Totally 15 (53.6 %) showed serological evidence of infection in either or both tests. Nine of 15 parents (60.0 %) and 8 out of 30 children (26.7 %) with significant rise in titre had antibody by CF and/or IEOP in the first drawn sera. The antibody titres in convalescent sera among these parents varied from 8 to 256 with a median of 64 by CF, and from 16 to 128 with a median of 32 by IEOP. The corresponding figures for children infected by reo-like virus were 8 to 64 with a median of 16 in both tests. There was a good

Table II. *Screening of faecal samples by IEOP, EM and tissue culture*

	IEOP (reo-like virus)		EM			Tissue culture	
			Positive		Negative	Positive ^a	Negative
	Positive	Negative	Reo-like virus	Other virus ^a			
Index cases	31	0	31	3	0	3	28
Siblings	4	23	5	3	20	3	24
Parents	0	59	0	2	57	1	58

^a Only adenovirus was found.

Table III. Serological evidence of infection by reo-like virus in 31 families, tested by CF (antigen NCDV) and IEOP (antigen human reo-like virus)

	No. of subjects tested	Significant rise in titre			No rise in titre
		CF	IEOP	CF and/or IEOP	
Index cases	29 ^a	24	23	27 (93.1%)	2
Siblings	8	2	3	3 (37.5%)	5
Parents	28	12	13	15 (53.6%)	13

^a Convalescent sera missing from 2 index cases.

correlation between IEOP and CF antibody titres in each serum specimen as shown in Fig. 1. The IEOP staining procedure increased the IEOP sensitivity to more than twice that seen without staining. A single precipitation line was observed in the test.

Relation between clinical and laboratory findings

Four out of 5 siblings who were excreting reo-like virus, also had symptoms of gastroenteritis, while one had a subclinical infection (Table IV). From 2 out of the 4 above-mentioned siblings paired sera were drawn, both with a significant titre rise. Further one child without symptoms had a significant rise in titre without shedding of virus. Among the siblings totally 6 out of 27 tested (22.2%) were either excreting virus or had a significant rise in titre. No etiological diagnosis could be established

in 7 cases of gastroenteritis among the siblings. Seven parents (25.0%) developed symptoms of gastroenteritis together with the serological response while 8 (28.6%) had a significant titre rise without any symptoms. As regards the parents with gastroenteritis, no etiological diagnosis could be established in 12 cases.

DISCUSSION

Earlier studies have shown that young children and infants who are infected with reo-like virus excrete the virus in faeces in measurable amounts during the first week of illness (1, 9, 10, 12, 14, 16, 17, 18). Furthermore, most children also respond serologically to the infection (2, 3, 9, 12, 14, 17). Recently, Kapikian et al. (10) showed that 32.5% of the parents of children with reo-like virus infection also responded serologically by CF at the time of the children's illness. In the study presented here the corresponding figure was even higher, 53.6%. Infections by reo-like virus among adults with excretion of virus have, until lately, only rarely been detected (6, 10, 15, 19, 20). In a preliminary report by von Bonsdorff et al. (5), however, 11 out of 42 gastroenteritis patients (26.2%) from the staff occupational health station were found to excrete reo-like virus.

In the present study, where totally 58 children and 59 parents were tested, all but one of the children with virus excretion also had a significant rise in titre, and had symptoms of gastroenteritis. One child, aged 10, showed serological response without symptoms and without shedding of virus. Among the parents, on the other hand, both symptom-free infections and infections with gastroenteritis symptoms, as indicated by significant titre rises in paired sera but without shedding of virus, were common. The fact that the exact day of onset of the

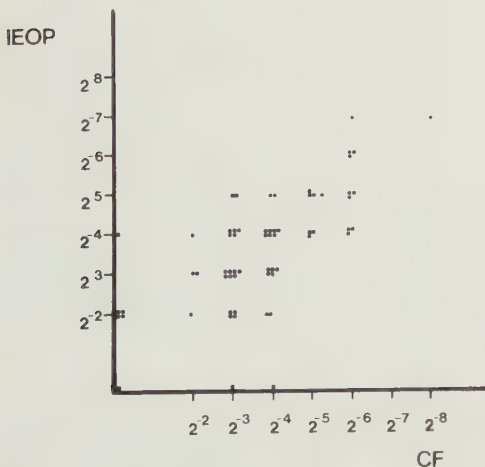


Fig. 1. Relation in titre between CF antibodies against the NCDV and precipitating antibodies against human reo-like virus among sera from 29 reo-like virus infected patients and 35 relatives.

Table IV. Clinical and subclinical reo-like virus infection among 31 families as shown by virus excretion and/or significant rise in titre (by CF and/or IEOP)

	Virus excretion		Significant titre rise	
	Gastroenteritis symptoms ^a	No symptoms ^a	Gastroenteritis symptoms ^a	No symptoms ^a
Index cases	31/31 (100%)	0/31 (0%)	27/29 (93.1%)	0/29 (0%)
Siblings	4/27 (14.8%)	1/27 (3.7%)	2/8 (25%)	1/8 (12.5%)
Parents	0/59 (0%)	0/59 (0%)	7/28 (25%)	8/28 (28.6%)

^a No. of cases/no. of subjects investigated.

parents' symptoms is unknown, and the faecal samples might have been collected rather late in the disease, might explain that no virus could be detected. Furthermore, the techniques used require 10^6 – 10^7 particles/ml of faecal suspension (18). Another possibility is that the parents had an earlier infection by the reo-like virus with a booster effect on the antibody titre by the present infection with no detectable excretion of virus. This assumption is supported by the fact that only 8 of 30 children (24.7%) with significant rise in titre had detectable antibody in the acute phase sera, while the corresponding figures for the parents were 9 out of 15 (60.0%). In addition, the mean titres in the convalescent sera in the two serological tests were 2–4 times higher among the parents, indicating a booster effect.

In our study, there was an extremely good correlation between CF and IEOP as regards the number of cases with significant rises in titre in paired sera or with the same level of antibody titres. Middleton et al. (12) found about 80% among adults possessing CF antibody, while only 3% had precipitating antibody. In a preliminary pilot study (19) on 20 healthy adults, we found that 60% and 55%, respectively, had CF and precipitating antibody. The discrepancy might depend on variations in the IEOP, e.g. in the staining technique used.

The present study has shown that the parents can be infected to a high extent, with or without symptoms, by the reo-like virus. Thus, the parents are involved in the spread of reo-like virus infections. Beside the transmission from child to adult, there is also a possibility for the parents to be a source of infection for children.

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Seasonal fluctuations in the occurrence of enterotoxigenic bacteria and rotavirus in paediatric diarrhoea in Addis Ababa

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This study (March 1977–February 1978) was performed at the Ethio-Swedish Pediatric Clinic, Addis Ababa, to determine whether there were any seasonal fluctuations in the occurrence of diarrhoea associated with enterotoxigenic enterobacteria (ETEB), rotavirus and two parasites (Giardia lamblia and Entamoeba histolytica).

A total of 1161 children (962 patients and 199 controls) were investigated. ETEB were isolated in 12.2% of the patients and 4.5% of the controls, rotavirus in 27.8% and 8%, and parasites in 6.8% and 1%, respectively. There is a statistically significant difference in the isolation rates between patients and controls ($P < 0.001$ for rotavirus, $P < 0.01$ for ETEB and parasites). Rotavirus was most prevalent in the 7–12 months age group and ETEB during the second year of life, while parasites showed a continuous increase with age.

Two peaks in the occurrence of ETEB were found during the year, the first in August (32.6%), the second in January (19.2%). Two peaks for rotavirus though not as distinct as for ETEB, were seen in June (42.7%) and November (36.4%). The isolation rate of parasites showed no consistent pattern during the year.

This study suggests a seasonal occurrence of ETEB and rotavirus but with no apparent correlation to climatological factors.

Diarrhoeal diseases are an important health problem and a prominent cause of infant mortality in developing countries. A community study in Addis Ababa (1) showed that children under two years of age were most affected. The average child in this age group experienced nine episodes of diarrhoea each year, which lasted a total of two months. Until recently the etiology of most of these episodes remained unexplained.

Although it was shown in the 1950s that strains of *Escherichia coli* may produce enterotoxin (2), these bacteria have been the subject of extensive research

only during the past decade. Other species of enterobacteria have also been shown to produce heat-labile enterotoxin(s) (3, 4). Enterotoxigenic enterobacteria (ETEB) are more prevalent as causes of infantile diarrhoea in countries with poor standards of hygiene (3–10).

During the 1970s, rotavirus, or human reovirus-like agent (HRVLA), was added to the list of presumptive enteropathogens of early childhood in developed as well as developing countries (4, 7, 11–13). *Giardia lamblia* and *Entamoeba histolytica* are, of course, also important enteric pathogens in young children.

In developed countries with a temperate climate, the prevalence of rotavirus shows marked seasonal variation, with a peak during the winter season (13–16). A few studies from developing countries with a subtropical or a tropical climate have also demonstrated seasonal variations, although there has not been any evidence of a clear correlation with climatic factors (17–19). A preliminary study in Addis Ababa suggested that there was an increase in the isolation rate of rotavirus at the beginning of the rainy season (20). The seasonal variation of ETEB infection has not been reported.

In Addis Ababa, more than 2000 m above sea level, the temperature is fairly constant throughout the year.

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A period of dry weather from October until February is usually followed by a period of light intermittent rains from March until May, which is then followed by heavy rains from June until September (21).

The aims of the present study were to determine the average relative frequencies of isolation of ETEB, rotavirus, and the two recognized enteric parasites (*Giardia lamblia* and *Entamoeba histolytica*) in infantile diarrhoea in a tropical setting, and to investigate any seasonal variation in the isolation rates over a whole year. A preliminary report on part of this study has already been published (20).

MATERIALS AND METHODS

Patients and controls

The study was conducted for one year, from March 1977 to February 1978, at the Ethio-Swedish Pediatric Clinic, Addis Ababa, Ethiopia. Approximately 100 000 attendances were recorded during the year, and it is estimated that 8000 children suffered from diarrhoea. No epidemic was seen during the year. A total of 962 patients were included in the study from among the children, mainly of a low socioeconomic background, attending the outpatient department, from Mondays to Saturdays, during regular working hours. This number of patients represents about 12% of all diarrhoea cases in children. Patients receiving concurrent antimicrobial therapy were excluded. A control group consisted of 199 children attending the child health clinic, but the controls were not perfectly age- and sex-matched. For the purposes of this study, diarrhoea was defined as four or more loose or mucoid stools, or more than one watery stool, per day.

Bacteriological investigations

Stool specimens were stored at 4 °C, and were subsequently sent to the National Bacteriological Laboratory (NBL) in Stockholm, Sweden, in a modification of the Stuart medium (22). Immediately upon arrival at NBL, within 10 days of collection, the specimens were cultured on Endo-agar plates and incubated for 18 h at 37 °C. Six colonies, if possible of different morphology, were picked from each Endo-agar plate. The isolated colonies were sub-cultured on 5% horse blood agar for 18 h for subsequent enterotoxin testing.

Enterotoxin testing. From March until November 1977, strains were inoculated from blood agar into brain-heart-infusion broth (Difco), one loopful in 10 ml of medium. In one batch of brain-heart-infusion broth an inhibitory effect was noticed that was probably caused by a ganglioside derived from the

brain tissue.^a Because of this, tryptone-yeast-extract broth was used from November onwards (23). The tubes were incubated at 37 °C on a shaker (100 rev/min) for 18 h and then centrifuged (4000 g) at 4 °C for 20 min.

The culture supernatants were assayed for heat-labile enterotoxin (LT) using the Y1 adrenal cell test. The adrenal cells in microtitration plates were exposed to 50 µl of the culture supernatants for 5 min, whereafter the cells were washed and fresh medium was added (24, 25). After 18 h the result was read independently by two people, the test being considered positive if 50% or more of the adrenal cells showed the typical rounded appearance.

Strains positive in the adrenal cell test were further tested for their ability to produce heat-stable enterotoxin (ST) using the infant mouse assay (26).

Virological investigations

Approximately 1–2 ml of faeces was stored in glass tubes at 4 °C and sent for virological examination to the Department of Clinical Virology, Malmö General Hospital, Malmö, Sweden. The specimens were tested by immunoelectro-osmophoresis for detection of rotavirus (27).

Parasitological investigations

Fresh stool specimens were examined under the light microscope (400x), with special reference to trophozoites of *E. histolytica* and *G. lamblia*.

Climatology

Climatological data were obtained from the National Climatological Service, Addis Ababa.

RESULTS

Altogether 1161 children were investigated, the monthly numbers ranging from 66 to 130. Of this total, 962 were children with diarrhoeal disease and 199 children without diarrhoea. Specimens were analysed for the prevalence of ETEB from only 931 patients and 178 controls because of lost samples. The age distribution of patients and controls is shown in Table 1.

Out of the 931 patients' specimens analysed, 114 specimens (12.2%) were found positive for ETEB (Table 2); 8 controls (4.5%) were also found positive, the difference being statistically significant (χ^2 -analysis, $P < 0.01$). Enterotoxigenic *Escherichia coli* (ETEC) were isolated from 109 (95.6%) of these

^a Bäck, E. Thesis, Karolinska Institute, Stockholm, 1979.

Table 1. Age distribution of patients ($n = 962$) and controls ($n = 199$)

Age	Patients with diarrhoea		Controls	
	No.	%	No.	%
0-6 months	288	29.9	104	52.3
7-12 months	289	30.0	30	15.1
13-24 months	242	25.2	28	14.1
25 months-4 years	95	9.9	16	8.0
≥ 4 years	48	5.0	21	10.5

Table 2. Frequency of isolations of different enteropathogens from faeces of patients with diarrhoeal disease

	Patients ($n = 962$)		Controls ($n = 199$)		<i>P</i> -value
	No.	%	No.	%	
ETEB	114 ^a	12.2	8 ^b	4.5	<0.01
rotaviruses	267	27.8	16	8.0	<0.001
parasites ^c	65	6.8	2	1.0	<0.01

^a 931 patients analysed.^b 178 controls analysed.^c Only findings of trophozoites of *G. lamblia* and *Ent. histolytica* are recorded.

114 patients. The LT positive strains from 41 patients were also tested for heat-stable (ST) enterotoxin and 22 (54%) were found positive.

Enterotoxigenic non-*E. coli* were isolated from 8 patients (0.9%). From 5 of these, non-*E. coli* were isolated in the absence of ETEC. Among the 8 controls, we found 4 cases (22%) with non-*E. coli* enterotoxigenic bacteria and in 2 cases we isolated non-*E. coli* only. The effect of the heat-labile enterotoxin of non-*E. coli* was neutralized by antiserum against cholera toxin and *E. coli* LT (28, and R. Möllby, unpublished data, 1979). Among the 14 non-*E. coli* isolates we found: 5 isolates of *Enterobacter cloacae* or *Enterobacter agglomerans*, 6 of *Citrobacter freundii*, 1 *Klebsiella pneumoniae*, 1 *Proteus morganii*, and 1 *Serratia marcescens*.

Rotavirus was detected in 267 (27.8%) of the patients and 16 (8%) of the controls (χ^2 -analysis, $P < 0.001$). Infection with ETEB and rotavirus at the same time occurred in 40 (4.3%) of the patients but not in any of the controls. Parasites were found in 65 patients with diarrhoea and in 2 of the controls (χ^2 -analysis, $P < 0.01$).

The relative frequencies of the enteropathogens in the patients of different age groups are shown in Fig. 1. The highest isolation rate of enterotoxigenic bacteria occurred during the second year of life (14.5%) while the highest isolation rate for rotavirus occurred in infants aged 7-12-months (31.1%). Infection with *Giardia lamblia* and/or *Entamoeba histolytica* showed a steady increase with age. Infection with two or three of these agents at the same time occurred most frequently during the second year of life (11.4%).

At the time of examination, the duration of the diarrhoea ranged from 1 day to more than 2 weeks with a median of 4 days. Approximately 10% had had diarrhoea of 2 weeks' duration or more on the day of examination. There was no apparent relationship between the duration and the etiology of the diarrhoea.

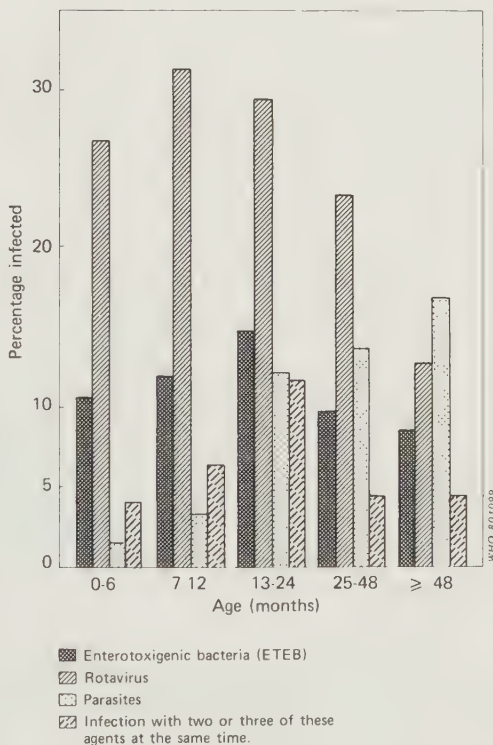
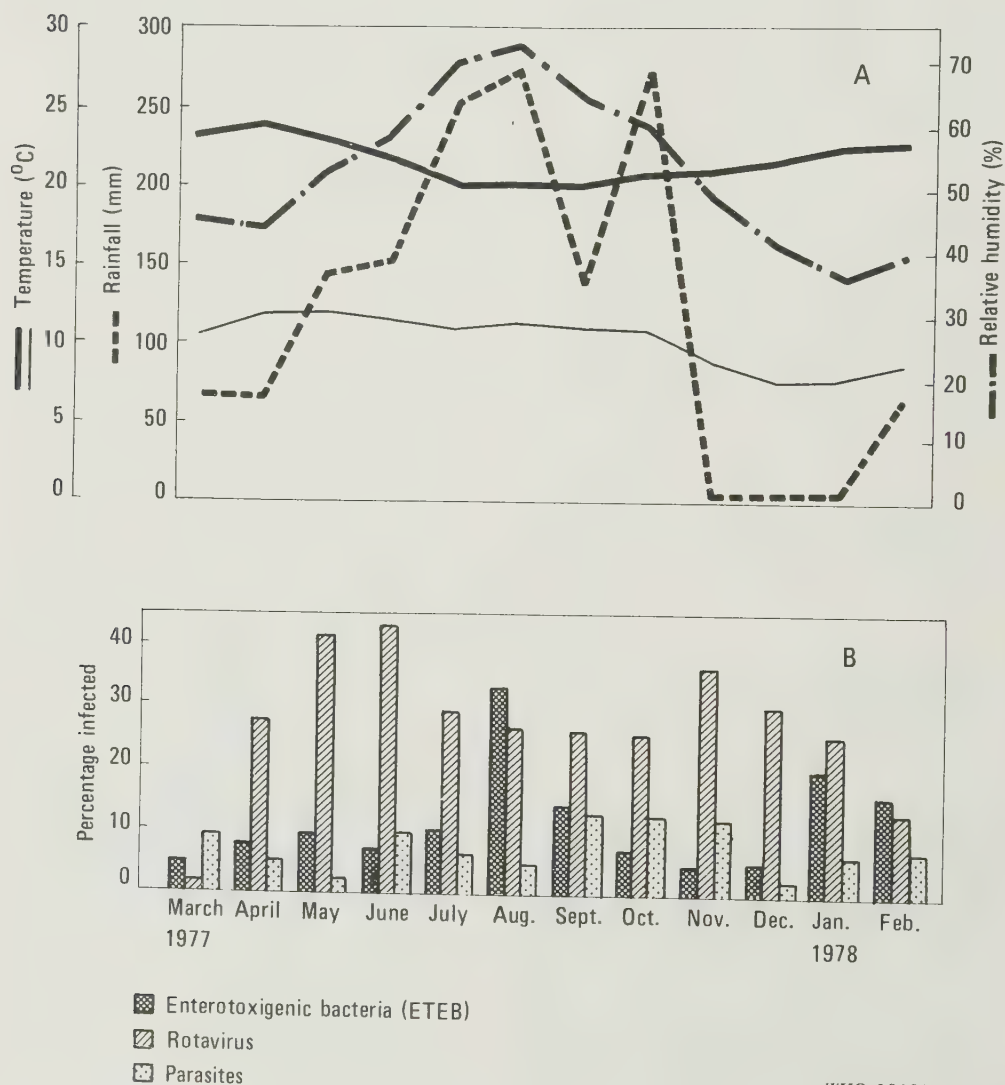


Fig. 1. Frequency of isolation of enteropathogens by age group. For parasites, only findings of trophozoites of *Giardia lamblia* and *Entamoeba histolytica* are recorded.

Data on rainfall, mean relative humidity, mean maximum and mean minimum daily temperatures for each month during the year of the study are shown in Figure 2A.

During the first five months of the study, enterotoxigenic bacteria were isolated infrequently (see Fig. 2B). In August, a definite peak was reached (32.6% of patients positive for ETEB) followed by a decrease



WHO 801098

Fig. 2. A. Climatic data. Monthly rainfall, mean relative humidity, and mean daily maximum and minimum night temperatures. B. Frequency of isolation of enteropathogens by month, March 1977–February 1978. For parasites, only findings of trophozoites of *Giardia lamblia* and *Entamoeba histolytica* are recorded.

until another but smaller peak (19.2%) occurred in January 1978. A similar pattern was seen for rotavirus isolations although the peaks occurred two months earlier (June, 42.7% of patients positive, and November, 36.4%) and were not as distinct as those for ETEB.

The frequency of isolation of parasites varied throughout the year, giving no evidence of a pattern similar to those of ETEB and rotavirus.

DISCUSSION

This study included more children than other similar studies and extended over a longer period. Thus, it was possible to study variation in isolation rates of the different pathogens over a 12-month period. However, to demonstrate seasonal variations conclusively, studies would have to extend over much longer periods.

In other studies the frequency of ETEB isolation has shown considerable variation depending on the epidemiological setting and may reflect genuine geographical differences (6-10, 20, 29, 30). However, some of the differences may be due to the different methods used for detecting heat-labile enterotoxin and different methods of interpretation.

In this study, a modification of the Y1 adrenal cell test described by Sack & Sack (25) was employed. This test is good for detecting heat-labile enterotoxin since there are few cytotoxic reactions and few false positive results (31). To avoid interpreting weak and non-specific cell reactions as positive, a test was regarded as positive only when 50% or more of the cells showed the typical rounded appearance.

We found ETEB producing heat-labile enterotoxin (LT) in 12.2% of the patients. Strains of ETEB from 41 patients were also tested for production of heat-stable (ST) enterotoxin (26), and it is possible that the number of patients with ETEB would have been somewhat higher if all LT-negative strains had been so tested. Data from a follow-up study indicate, however, that infections caused by ETEB producing

only heat-stable toxin were rare in this population (G. Stintzing, et al., unpublished data, 1978).

Earlier studies (8-10) indicate that isolation of heat-labile enterotoxigenic *E. coli* is not *a priori* evidence that this organism is the etiological agent. In this study, not unexpectedly, ETEB was also isolated from some, although significantly fewer, of the controls.

In addition to ETEB, rotavirus, and the two parasitic species, other bacterial pathogens were also studied in these outpatients during limited periods of the year. A preliminary report (20) showed an average isolation frequency of 5.6% for enteropathogenic *E. coli* (EPEC) and of 4.4% for *Shigella* species. During this period, March until July, neither salmonellae nor vibrios were isolated, and *Yersinia enterocolitica* type 3 was isolated in only one case. This is in agreement with previous studies in from Addis Ababa (3, 32, 33). A later study has shown that *Campylobacter fetus jejuni* is also an important etiological agent in this population (A. Thorén, unpublished data, 1979).

Rotavirus was isolated from 27.8% of the patients, a proportion comparable with those found in other developing countries (10, 12, 19, 29). Also the age distribution of patients with rotavirus infections was similar to that reported from other studies (13-16, 34). As the proportion of rotavirus-positive specimens among the controls was higher than in earlier studies (9, 10, 14, 17, 19, 29, 30), special attention was paid to these samples. All 16 positive samples were shown to contain rotavirus by electron microscopy. This high rate might reflect the difficulty of finding suitable control subjects without any known recent history of diarrhoea, especially among infants aged 0-6 months, this being the group from which most of the control patients were obtained.

The frequency of isolation of ETEB showed two peaks, in August and January, but these did not seem to be related to any climatological factor. Two peaks were also seen in isolations of rotavirus, and these preceded the peaks for ETEB by two months. The findings of this study did not suggest any seasonal fluctuations in the occurrence of *Giardia lamblia* or *Entamoeba histolytica*.

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RÉSUMÉ

FLUCTUATIONS SAISONNIÈRES DANS L'APPARITION DE BACTÉRIES ET DE ROTAVIRUS ENTÉROTOXIGÈNES
AU COURS DES DIARRHÉES DE L'ENFANT À ADDIS-ABÉBA

Une étude longitudinale sur les enfants présentés à la consultation externe du dispensaire pédiatrique éthiopo-suédois d'Addis-Abéba pendant un an (mars 1977-février 1978) a été réalisée pour déterminer s'il existait des fluctuations saisonnières dans l'apparition des diarrhées liées à la présence de bactéries entérotogènes (ETEB), de rotavirus et de deux parasites (*Giardia lamblia* et *Entamoeba histolytica*).

L'étude a englobé 1161 enfants au total (962 malades et 199 témoins). Des échantillons de selles ont été examinés à l'état frais en recherchant spécialement les trophozoïtes de *E. histolytica* et de *G. lamblia*. Puis les échantillons ont été envoyés au Laboratoire national de Bactériologie dans du milieu de Stuart modifié. Dans les dix jours qui ont suivi la collecte, six colonies provenant de chaque malade ont été soumises à la recherche d'entérotoxines thermolabiles (LT). Le surnageant des cultures a été éprouvé à l'aide de cellules surrénales Y1. Dans les isolements renfermant des LT, on a encore recherché la présence d'entérotoxines thermostables (ST) par étude sur le souriceau. Les rotavirus ont également été recherchés dans certains échantillons par une technique immunoelectroosmographique.

Des ETEB ont été isolées chez 12,2% des malades et 4,5% des témoins, des rotavirus chez 27,8% et 8%, et des parasites chez 6,8% et 1% respectivement. Il y a une différence statistiquement significative dans les taux d'isolement entre les malades et les témoins ($P < 0,001$ pour les rotavirus, $P < 0,01$ pour les ETEB et les parasites). Des infections simultanées à ETEB et à rotavirus se sont produites chez 4,3% de l'ensemble des malades. Des *Escherichia coli* entérotogènes (ETEC) ont été isolés chez 95,6% des malades atteints par des ETEB, et 54% des malades avaient des souches produisant également l'entérotoxine thermostable

(ST). Des germes entérotogènes autres que *E. coli* (ils appartenaient aux espèces *Enterobacter*, *Citrobacter*, *Klebsiella*, *Proteus* et *Serratia*) ont été isolés chez 7% de tous les sujets atteints de ETEB.

La prévalence des rotavirus était la plus grande dans le groupe d'âge de 7-12 mois et celle des ETEB au cours de la seconde année de vie, tandis que les parasites augmentaient de façon continue avec l'âge. La durée de la diarrhée au moment de l'examen allait de un jour à plus de deux semaines avec une médiane de quatre jours. Il n'y avait pas de relation manifeste entre la durée et l'étiologie de la diarrhée.

Au cours de l'année, le taux d'isolement des ETEB a présenté deux pics, le premier en août (32,6%), le second en janvier (19,2%). Deux pics ont été observés pour les rotavirus, quoique moins nettement que pour les ETEB, le premier en juin (42,7%) et le second en novembre (36,4%). Le taux d'isolement des parasites n'a montré aucune homogénéité au cours de l'année. Les deux premiers pics de ETEB et de rotavirus sont apparus pendant les mois de fortes pluies, d'humidité relative élevée, et alors que l'écart entre les températures moyennes maximales et minimales, diurnes et nocturnes, était faible. Les deux pics suivants ont été observés durant des mois où il n'est pratiquement pas tombé de pluie, où l'humidité relative était faible et où l'intervalle entre les températures diurnes et nocturnes extrêmes était grand.

On peut donc conclure que les rotavirus et les ETEB ont été, semble-t-il, d'importants agents étiologiques des diarrhées infantiles à Addis-Abéba. Cette étude est en faveur d'une apparition saisonnière sans aucune corrélation manifeste avec les facteurs climatiques.

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Detection of a Human Rotavirus Strain Different From Types 1 and 2—A New Subgroup? Epidemiology of Subgroups in a Swedish and an Ethiopian Community

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A possible new subgroup of human rotavirus was found by crossed immunoelectrophoresis and enzyme-linked immunosorbent assay. The epidemiology of the established subgroups 1 and 2 and this new variant was studied in two different communities. Of 398 rotavirus isolates from Malmö, Sweden, 26.8% were of type 1, 71.7% of type 2, and 1.5% of the new variant. Corresponding figures for 384 samples from Addis Ababa, Ethiopia, were 33.1% type 1 and 66.9% type 2. A total of 87% of the Swedish and 79% of the Ethiopian rotavirus-positive samples could be classified. The yearly distribution of the subgroups was stable and similar in Sweden and Ethiopia. The new variant could only be found in one outbreak during 1979. Among children with sequential infections eight of ten primary infections were type 1, and no one shed the same type of rotavirus twice.

Key words: rotavirus, subgroups, ELISA

INTRODUCTION

Rotavirus is a well-known agent of gastroenteritis in many parts of the world (for review see Steinhoff [1980]). At least two different subgroups of rotavirus have been distinguished by complement-fixation, immunoelectronmicroscopy, enzyme-linked immunosorbent assay (ELISA) and immune adherence hemagglutination [Zissis et al, 1978; Yolken et al, 1978; Kapikian et al, 1981]. Further subgroups are expected to be found, judging from the findings by polyacrylamide electrophoresis of ribonucleic acid segments

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from rotavirus [Kalica et al, 1978]. Different serotypes within the subgroups have been detected by plaque reduction assay [Wyatt et al, 1982].

In an outbreak in Malmö, Sweden, a group of rotavirus strains were found, which were nontypable by available antisera by ELISA.

The aim of this study was to characterize this variant, establish its possible spread, and compare its distribution with the recognized subgroups in two different sets of samples.

MATERIALS AND METHODS

Stool Samples

The study was performed on suspended stool samples from two different groups of patients with rotavirus gastroenteritis, diagnosed by routine testing by ELISA. One group totalling 455 rotavirus-positive samples was obtained from the pediatric clinic, Malmö General Hospital; 90 of the samples were collected in 1977, 93 in 1978, 95 in 1979, 98 in 1980, and 79 in 1981. The other group of samples was collected at the Ethio-Swedish Pediatric clinic, Addis Ababa, Ethiopia [Stintzing et al 1981; Thorén et al, 1982] and consisted of 485 rotavirus-positive samples. Of these 221, 125, and 139 were collected in 1977, 1978 and 1979, respectively. The samples from 1979 were obtained from children admitted to hospital with severe gastroenteritis which had lead to dehydration. The samples were sent refrigerated to Sweden by air. All fecal suspensions were treated according to recognized procedures at the laboratory [Tufvesson and Johnsson, 1976].

Rotavirus Antigens for Immunisation

Strains of types 1 and 2 (kindly typed by G Zissis, Bruxelles) were purified according to the method of Petric et al [1975].

Preparation of Antisera

Rabbit rotavirus antibody used for capturing rotavirus antigens was raised as described by Middleton et al [1977]. Type-specific antibody was produced in guinea pigs. Injections with purified type 1 and 2 virus were performed according to a scheme described earlier [Tufvesson and Johnsson, 1976]. Final preparation of the type specific antiserum was carried out by affinity chromatography according to Johansson and Wadell [1978].

Subgrouping by Enzyme-Linked Immunosorbent Assay (ELISA)

Microtiter plates were coated with antibody and stored at +4°C overnight. After washing, 50 µl of each fecal suspension was added in duplicate and incubated for two hours. After a second wash, the plates were incubated at 37°C for two hours with the type-specific antibodies. Four antibody units of each of the type-specific antibody was used. One antibody unit was defined as the last dilution giving a distinct reaction with homologous virus by ELISA.

Reaction between antibody and antigen was detected by incubation for two hours with horseradish peroxidase conjugated rabbit antiguinea pig IgG antiserum (Cappel laboratories), followed by 15 minutes' incubation with enzyme substrate (ortophenylenediaminehydrochloride and hydrogen peroxide). The reading was performed in a Titerc Multiscan spectrophotometer at 492 nm.

Antigens with known subgroup showed a distinct reaction with the corresponding antibody, with an extinction exceeding 1.0. Reaction with antibodies against the other subgroup was less than one fourth of the homologous reaction. A type-specific reaction was thus defined as a reaction which was at least four times greater than the other.

Crossed Immunoelectrophoresis

Rotavirus strains were purified and ultrasonicated before the analysis. Four strains of type 1 and nine strains of type 2 were tested. These were compared to two new strains. As antibody, pooled human IgG which contained antirotavirus antibody (Kabi, O.165g/ml) was used. The test was carried out according to a scheme for crossed immunoelectrophoresis described by Weeke [1973]. Briefly, 10 ml of 1% agarose was pipetted onto a glass plate (95 × 85 mm), and 10 μ l of each sample was applied into punched wells in the gel. Electrophoresis in the first dimension was run for 90 minutes with 10 V/cm. Afterward the gel was sliced and the strips with separated antigens were applied to new plates. Eight milliliters of antibody-containing gel was then poured onto the remaining part of the plate. Electrophoresis in the second dimension was then performed overnight with 2 V/cm. For the electrophoresis a LKB Multiphor, which permits three plates to be processed at the same time under equal conditions, was used. After electrophoresis the gels were pressed, washed, dried, and stained with Comassie brilliant blue before examination.

RESULTS

Characterization of Subgroups 1 and 2 by Crossed Immunoelectrophoresis

The specimens tested reacted with the antibody at two different positions. Samples of the two different subgroups tested in the same electrophoretic run all had one peak situated in the same position, probably representing a common antigen. The other peak, however, differed between the types and evidently represented a type-specific reaction. The patterns are illustrated in Figure 1B. The average distances between the common antigen and the type-specific antigen were as follows: type 1, 6 mm (range 5–7 mm); type 2, 13 mm (range 12–13 mm).

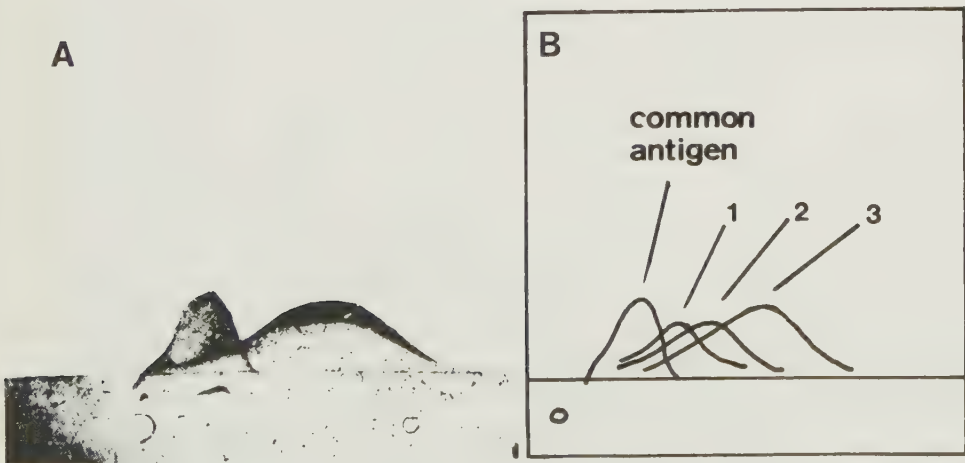


Fig. 1. Mobility pattern of rotavirus subgroups in crossed immunoelectrophoresis. A) Type 3 rotavirus on stained electrophoresis plate. B) Replication showing the different patterns of the types.

Definition of a Type Different From 1 and 2

A group of nine specimens, originating from a distinct outbreak at a pediatric ward in Malmö, could not be typed by ELISA using antisera against type 1 and 2. Two of these specimens were compared by crossed immunoelectrophoresis to the strains of type 1 and 2. The same reaction as regards the common antigen could be shown for these two strains, while the position of the type-specific reaction differed (Fig. 1A, B). The distance between the peaks was measured to 19 mm. Guinea pigs were immunized with purified antigen from these two samples, and specific antisera were prepared according to the above-mentioned procedures. Six of the nine samples could now be shown to share this type of antigenicity with a type-specific reaction in ELISA. This pattern will be called type 3.

Epidemiology of Subgroups

Out of the total 455 rotavirus-positive samples from Malmö General Hospital, 398 (87%) could be typed. The overall distribution of subgroups was 107 (26.8%) type 1, 285 (71.7%) type 2, and 6 (1.5%) type 3. The distribution of subgroups for each year of the survey is shown in Table I. There was little variation between the years. Type 2 dominated each year, while type 1 accounted for about 25% yearly. Type 3 strains were only found in the six cases from 1979 previously mentioned.

Among the samples tested above, 20 were obtained from patients with multiple infections. The subtypes of these samples can be seen in Table II. Of the ten patients, eight suffered from a type 1 primary infection. Types 2 and 3 caused one primary infection each. Nine out of ten secondary infections were of type 2. No patient shed virus of the same subgroup on both occasions. The primary infections occurred when the children were 3–8 months old, while their secondary infections occurred at 11–34 months of age.

The overall distribution of subgroups among the Ethiopian samples, where 384 (79%) could be typed, was 127 (33.1%) type 1 and 257 (64.9%) type 2; no samples of the third type were identified. Table III presents the yearly figures. In samples from 1977 and 1978 there is an even distribution of the types. Among the samples from 1979, taken

TABLE I. Yearly Distribution of Rotavirus Subgroups Among Samples From Malmö General Hospital

	1	2	3
1977	21(28) ^a	54(72)	—
1978	23(27)	62(73)	—
1979	29(31)	60(63)	6(6)
1980	19(24)	60(76)	—
1981	15(23)	49(77)	—

^aFigures within parentheses are percentages.

TABLE II. Rotavirus Subgroups Among Children With Multiple Infections

Children tested	Primary infection: Type			Secondary infection: Type		
	1	2	3	1	2	3
10	8	1	1	1	9	—

TABLE III. Yearly Distribution of Rotavirus Subgroups Among Samples From Addis Ababa

	1	2	3
1977	63 (36) ^a	112 (64)	—
1978	35 (35)	64 (65)	—
1979	29 (26)	81 (74)	—

^aFigures within parentheses are percentages.

TABLE IV. Distribution of Subgroups According to Age

Age (months)	Addis Ababa 1977-78 (outpatients)			Addis Ababa 1979 (hospitalized patients)			Malmö (hospitalized patients)		
	1	2	3	1	2	3	1	2	3
0-6	40 (33) ^a	82 (67)	—	14 (32)	50 (68)	—	31 (33)	62 (66)	1 (1)
6+	58 (38)	94 (62)	—	15 (23)	51 (77)	—	76 (25)	223 (73)	5 (2)

^aFigures within parentheses are percentages.

from hospitalized patients with severe gastroenteritis, no significant difference in the overall distribution of subgroups could be detected.

In Table IV, the patients are grouped according to age. The Ethiopian samples from 1979 are in a separate group, as they come from more seriously ill and hospitalized patients. Among the youngest patients there is no difference in subgroup distribution. However, among children older than six months both hospitalized Swedish and Ethiopian children have a similar pattern with lower incidence of type 1 infections than the less-ill Ethiopian outpatients (Ψ^2 test, $P < 0.05$).

DISCUSSION

Subgrouping of fecal samples positive for rotavirus has been carried out by complement-fixation, immunoelectron microscopy, ELISA and immune adherence hemagglutination [Zissis et al, 1978; Yolken et al, 1978; Kapikian et al, 1981]. This study confirms that the ELISA technique is suitable for this purpose, especially when large numbers of samples are being tested. Immunoelectron microscopy is not comparable to this technique as it demands much more work per sample. However, it is important to realize that a typing technique, where the identification of different types is defined as greater reactivity with one reference serum than with others, does not make it possible to distinguish any new types which are in some way related to the predefined ones.

By crossed immunoelectrophoresis it was possible to demonstrate two different types of reactions between rotavirus and antibody, a common reaction and a type-specific. This pattern has also been described by Grauballe et al [1979]. By comparing the profile of different strains a new pattern of antigenicity of two strains could be demonstrated. Antibody against these strains was used for typing by ELISA, and this made it possible to detect a total of six strains representing this variant. Whether this new type is a transient or persistent phenomenon could not be evaluated in this investigation. However, the occurrence of antibody against this type in pooled human IgG indicates that the type can be more common in the community than could be seen in this material.

Yolken et al [1978] found a considerable variation in the yearly distribution of types. This could not be confirmed in this study. It seems likely that in Sweden there is a more stable distribution, where type 1 accounts for about 25% yearly and type 2 for about 75%. Regarding the Ethiopian samples the distribution also seems stable, although there is a higher prevalence of type 1 infections among outpatients older than six months.

The falling frequency of type 1 among the children older than six months compared to younger age groups, may suggest a difference in the appearance of the different types. The same conclusion may be drawn from the group of children with sequential rotavirus illness, where the primary infections, occurring at ages 3–8 months, were type 1 in eight out of ten cases. On the other hand, it has been stated [Yolken et al, 1978] that in spite of the difference in distribution of the subgroups of isolated virus strains, antibodies against type 1 and type 2 virus are almost equally distributed among children of all ages, implying that infections of both types are equally common, but type 2 infections more often produce clinical symptoms.

It is also interesting to note that among the children who had more than one rotavirus infection no one shed the same type on both occasions. This could imply that although rotaviruses share a common antigen, only a type-specific immunity is developed during the infections.

The rotavirus-positive samples that could not be typed were generally samples with a weak reaction already in the routine detection of the virus, which suggests that the typing procedure has a lower sensitivity. It has also been noticed that repeated freezing and thawing of the samples reduces the reaction in the typing procedure. Thus, the ideal approach for the typing by ELISA is immediate processing of fresh samples.

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VI

A PROSPECTIVE STUDY OF ROTA VIRUS INFECTIONS IN NEONATAL AND MATERNITY WARDS

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Running title: Neonatal rotavirus infections

ABSTRACT

The occurrence and symptomatology of rotavirus infections were studied at three maternity wards and one neonatal unit. Rotavirus was identified in 12.7 % of 553 infants and 1.3 % of 542 mothers at the maternity wards. Infections were more frequent in a mixed obstetric/gynecology ward than in the pure obstetric wards. Only 10 % of the infants had symptomatic infections. Subgroups of rotavirus was determined in 41 infants: 22 of subgroup 1 and 19 of subgroup 2, which is the subgroup accounting for the majority of childhood gastroenteritis. Rotavirus was found in faecal samples from 37 % of the infants at the neonatal unit during an eight month survey. A seasonal variation with most infections during colder months was seen. Subgroup determination was possible in 29 cases, 14 subgroup 1 and 15 subgroup 2. Fifteen per cent of the infections demonstrated diarrheal symptoms. No significant difference among other clinical data registered was seen among rotavirus infected compared to the non-infected infants. We conclude that neonatal rotavirus infections occur as an endemic infection at our maternity wards possibly combined with infections due to external sources of virus in mixed wards and neonatal units.

INTRODUCTION

Rotavirus infection is the most common cause of gastroenteritis in children between the ages of 6 months and 2 years (16). Neonatal infection may follow a quite different, often mild or asymptomatic course (3,4,5,11,13,14). An association with more severe diarrhea, including necrotising enterocolitis, has, however, also been suggested (6).

Two subgroups of rotavirus have been described (17,20,21), and recently a tentative third subgroup was detected (9,17). Subgroup 2 seems to be the predominant cause of childhood gastroenteritis (17,18). There is a difference among strains encountered in the community to those appearing in nurseries and special care baby units, as seen by polyacrylamide electrophoresis (12).

The present study was undertaken to ascertain the incidence, subgroup specificity and symptomatology of rotavirus infections among neonatals at Malmö General Hospital.

PATIENTS AND METHODS

Patients: Infants born between January 1st and March 31st 1983 at the maternity wards of Malmö General Hospital and their mothers were studied. One faecal specimen for virological examination was collected within 7 days after birth

from each of 553 infants and 542 mothers. The second part of the study comprised all infants admitted to the neonatal unit of the paediatric clinic between January 1st and August 31st 1983. 150 infants delivered faecal specimens once a week until discharged. Characteristics concerning birth weight and gestational age are given in Table 1.

Maternity wards: Two of the wards, each with 20 beds, functioned only as obstetric wards, while the third was a mixed obstetric/gynecology ward with 17 beds, of which 6 were reserved for obstetric patients.

Neonatal unit: One ward with a capacity of 20 patients, connected to the paediatric clinic.

Clinical observations: The occurrence of diarrhea and vomiting among all patients was registered. Overtemperature, undertemperature, elevated levels of C-reactive protein, or increased leucocyte particle count among patients at the neonatal unit are accounted for as signs of infections. Antibiotic treatment was registered. Neonatal disturbance of breathing and pneumonia were registered, and are accounted for as respiratory problems. Requirement of incubator care was also registered.

Detection of rotavirus and rotavirus subgroups

Suspensions were made from the faecal samples according to previous descriptions (16). Rotavirus in the suspensions was detected by a modification of the ELISA-test described by Yolken et al (19). Microtiter plates were coated with 100 μ l of rabbit rotavirus antibody (17). After washing, 75 μ l of faecal suspension was added and incubated for 90 min at 37°C. Antibody-rotavirus reaction was detected by incubation for 90 min with horse-radish peroxidase conjugated rabbit rotavirus antibody followed by 15 min incubation with enzyme substrate. Reading was performed in a Titertec Multiscan spectrophotometer at 492 nm. A blocking test was performed on positive samples to confirm specificity of the reaction (19).

Samples from the maternity wards designated as rotaviruspositive were also tested by electron microscopy according to previous descriptions (16).

Subgroup determination was performed by a previously described ELISA-test (17).

RESULTS

1. Maternity wards

Rotavirus was identified totally in 70/553 (12.7 %) of the infants at the maternity wards. The infection rate differed between the wards with only obstetric patients, where 11.6 % of the infants were excreting rotavirus, and the mixed ward, where 25.6 % excreted virus. No variation in distribution over time was seen at the different wards. Only 7/70 (10 %) of the rotavirusinfected infants demonstrated mild symptoms of gastroenteritis.

Among the mothers 7/752 (1.3 %) were excreting rotavirus, all asymptomatic. In six of these cases, rotavirus was also demonstrable in the faecal samples from their infants.

Using electron microscopy, it was possible to demonstrate rotavirus in 45/70 (64.3 %) of the ELISA positive samples from the children, while 2/7 (28.5 %) samples from the mothers also were positive by electron microscopy.

Subgroup determination was possible in 41/70 (58.6 %) of the samples from the infants. Totally 22 (53.7 %) were of subgroup 1 and 19 (46.3 %) of subgroup 2. The distribution at the different wards is shown in Table 2.

All samples from the mothers excreting rotavirus were of subgroup 2. The 6 infants of these mothers, who were excreting rotavirus were also of subgroup 2.

2. Neonatal unit

Rotavirus was detected in 55/150 (37 %) infants nursed in the neonatal unit. The monthly distribution, with a peak in incidence during the colder months, and falling frequency during late spring and summer is seen in Table 3. During August, however, an increasing incidence was seen. Virus excretion lasted for 7 days - 6 weeks among the patients.

In 27 (49 %) of the rotaviruspositive patients at least the initial sample taken at the neonatal unit was negative. During January - March, when all infants also were screened at the obstetric unit, four out of totally 30 (13 %) rotaviruspositive infants were infected already at the maternity wards.

The clinical features of the rotavirus excreting infants are compared to non excreting infants in Table 4. Diarrhea was seen in 8 (15 %) of the rotaviruspositive infants, compared to 3 (3 %) among rotavirusnegative, all having only

mild symptoms. The difference is significant (Fishers exact test, $p < 0.01$). No significant differences were found between the groups concerning other clinical data recorded. A low birthweight (< 2.500 g) was seen in 42 % among rotavirus-positive infants compared to 23 % among rotavirusnegative (χ^2 -test, $p < 0.05$). A gestational age of less than 37 weeks was seen in 47 % among rotaviruspositive infants compared to 27 % among rotavirusnegative (χ^2 -test, $p < 0.05$) (Table 1).

The proportion of rotavirusinfected infants increased from 21 % among infants treated less than one week to 70 % among infants with more than 3 weeks treatment (Table 4).

Subgroup determination was possible in 29/55 (53 %) of the rotaviruspositive samples. The distribution was 14 of subgroup 1 and 15 of subgroup 2 (Table 2).

DISCUSSION

Neonatal rotavirus infections were originally described by Chrystie et al (3), and have later been seen during longer periods in some major centres (1,4,5,13), indicating endemicity of the infection. In our study, the constant frequency of infections at the obstetric wards, contrasting to the seasonal variations seen among older children with symptomatic infections (16), indicate endemicity of the neonatal infections. Furthermore, the distribution of subgroups at the two wards, used only for obstetric patients, differed from what is seen in the community (17,18), possibly a reflexion of an endemic circulation in the wards of rotavirus strains of subgroup 1. The mothers of the infants were the source of infection in 1 % of the cases. In the mixed ward a higher incidence of infections and a common distribution of subgroups are implications of an outside source in the majority of the infections, such as visitors to the ward. In fact, the regulations for visitors differ, as a principal prohibition of visits for others than the close family exists at the obstetric wards.

A high incidence of rotavirus infections was also seen in the neonatal unit with a subgroup distribution similar to the two maternity wards. Totally 49 % of the rotaviruspositive infants in the study had at least one negative sample in the beginning, indicating an endemic source of the infection. New virus was also continually added to the unit by already infected infants transferred from the maternity wards. In this study 13 % of the rotavirusinfected patients during the peak season were infected already at the maternity wards. A similar behaviour of rotavirus infections has been demonstrated in a piglet nursery, with an increase in severity of diarrhea whith prolonged use of the nursery

(10). On the other hand, the monthly distribution of infections at this unit coincides with the distribution of symptomatic rotavirus infections among older children (16), which points to a possible outside source of infections in analogy with the mixed gynecology/obstetric ward.

One previous study states the most important factors influencing neonatal rotavirus infections are proximity to other newborns and handling of the infants by adults, not in the close family (1). Once rotaviruses become established in a nursery, they are difficult to eradicate, and thus, a transfer from infant to infant via the hands of visitors, personnel and contaminated material is almost impossible to prevent (7). Hospitalization time was in this study found to be an important factor determining the risk of infection, with an increased proportion of infants infected from 21 % to 70 % after respectively one and three weeks nursing. This also explains the high rate of rotavirus infections seen among prematurely born, and thus, infants of low birth-weight, who have the longest periods of hospitalization.

Studies of neonatal rotavirus infections have indicated severe, mild and asymptomatic cases (4,6,14). In our study, 10 % of the infections at the maternity wards, and 15 % of the infections in the neonatal unit had mild gastroenteritis symptoms. Regarding other clinical features, no difference was seen between the groups in this study. The protection from severe rotavirus illness can be conferred by maternal antibody reaching the embryo via the placenta as well as colostrum containing antibody against rotavirus (15).

A prolonged virus excretion up to 6 weeks was seen among neonatally infected children. Thus, neonatal infection is of importance, providing a source of spreading the infection over a long period. On the other hand, the infection can be of benefit for the infant, although not conferring immunity against reinfection, at least protecting against the development of clinically severe disease during reinfection in childhood (2).

The specificity of ELISA for the detection of rotavirus in neonates has been questioned (8). However, in the study, it was found specific as indicated by the blocking test with no false positives, and sensitive, as indicated by an excess number of detected cases compared to electron microscopy. Subgroup determination with our and comparable methods among older children with symptomatic infections has been possible in 70-80 % of the samples (9,17,20). The lower figures among the neonates in this study can depend on a lower virus

excretion, samples taken with rectal swabs and thus, leaving less material, or factors in the faecal samples interfering with the test.

In summary, our findings suggest that neonatal rotavirus infection occurs mainly as an endemic infection in the obstetric wards. In the mixed obstetric/gynecology ward and the neonatal unit endemic infections and outside sources of infection seem to be of equal importance. Neonatal rotavirus infections are as a rule asymptomatic.

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	rota positive (N = 55)	rota negative (N = 95)
birth weight $\leq$ 2 500 g	23 (42 %)	22 (23 %)
$>$ 2 500 g	32 (58 %)	73 (77 %)
gestational age (weeks)		
$<$ 30	1 (2 %)	1 (1 %)
30-36	25 (45 %)	25 (26 %)
37-42	28 (51 %)	67 (71 %)
$>$ 42	1 (2 %)	2 (2 %)

Table 1. Characteristics of 150 children treated at the neonatal unit

	subgroup 1	subgroup 2	subgroup not identified
maternity ward I	11	8	14
II	10	5	11
mixed ward	1	6	4
neonatal unit	14	15	26

Table 2. Number of patients excreting different subgroups of rotavirus at the maternity wards and the neonatal unit

	rotavirus infected/total number nursed
January	8/15 (53 %)
February	8/14 (57 %)
March	14/20 (70 %)
April	8/25 (32 %)
May	4/20 (20 %)
June	2/20 (10 %)
July	1/15 (7 %)
August	10/21 (48 %)

Table 3. Monthly distribution of rotavirus infections of the neonatal unit

	rota positive (N = 55)	rota negative (N = 95)
diarrhea	8 (15 %)	3 (3 %)
signs of infection	29 (53 %)	43 (45 %)
antibiotic treatment	22 (40 %)	20 (21 %)
respiratory problems	8 (15 %)	18 (19 %)
incubator care	16 (29 %)	12 (13. %)
hospitalization time < 1 week	19	73 (21 %)
1-3 weeks	21	16 (57 %)
> 3 weeks	15	6 (70 %)

Table 4. Clinical features among patients at the neonatal unit

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